Study of apoptosis and phagocytosis in the absence of tissue transglutaminase
Role of extracellular matrix-related proteins in the growth and migration of oral keratinocytes

UNIVERSITY DOCTORAL (Ph.D.) THESIS

by
Zsolt Sarang

Supervisors: Prof. Zsuzsa Szondy and
Prof. Roland Grafström

UNIVERSITY OF DEBRECEN
Medical and Health Science Center
Faculty of Dentistry
DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

DEBRECEN, 2005

Table of content

Prologue

During my Ph.D. period at the Department of Biochemistry and Molecular Biology in Debrecen I spent two years in the Institute of Environmental Medicine at Karolinska Institute, Sweden where I was studying the culture and physiology of primary and transformed human buccal keratinocytes under the supervision of Prof. Roland Grafström. According to this, my dissertation consists of two separate sections: the first part compromises my work completed at the Department of Biochemistry and Molecular Biology; the second part contains experiments carried out at Karolinska Institute.

List of abbreviations

ALT	Alanine aminotransferase
ADR	α_1-b-adrenergic
AST	Aspartate aminotransferase
CEC	Chloroethylclonidine
CFE	Colony-forming efficiency
COL	Collagen
DXM	Dexamethasone-acetate
ECM	Extracellular matrix
FCS	Foetal calf serum
FLIP	FLICE-inhibitory protein
FN	Fibronectin
IL	Interleukin
ITG	Integrin
LTBP	Latent TGF-β binding protein
NOK	Normal oral keratinocytes
PBS	Phosphate buffered saline
PLC	Phospholipase C
PS	Phosphatidylserine
SCC	Squamous cell carcinoma
TCR	T cell receptor
TGase	Transglutaminase
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha

Part I

Introduction

Apoptosis in general

In eukaryotic organisms, two distinct ways of cell death are discriminated: necrosis and programmed cell death (PCD) (Fig. 1). Necrotic cell death occurring upon tissue injury is a passive process. The damaged cell is enlarged, finally the plasma membrane disrupts, and cytosolic components are released into the extracellular space, causing an inflammatory reaction. The two major forms of PCD are terminal differentiation and apoptosis. The name „apoptosis" was first introduced by Kerr, Wyllie and Currie (Kerr JF *et al*., 1972). Apoptosis is crucial for tissue homeostasis in multicellular organisms. It is an active reaction and plays an important role in many physiological processes, especially in the immune system, in the nervous system, and in development (Krammer PH, 1999; Vaux DL and Korsmeyer SJ, 1999).

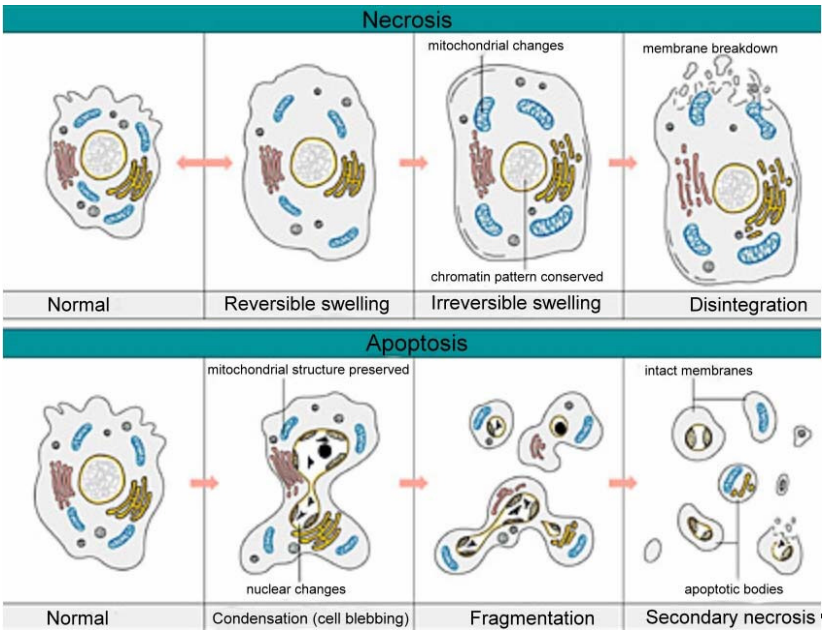

Figure 1 Morphological changes during necrosis and apoptosis

Morphology of apoptosis

Apoptosis occurs in a well-organized sequence of morphological events (Kerr JF *et al.,* 1972). The cell first undergoes nuclear and cytoplasmic condensation with blebbing of the plasma membrane and exposure of phosphatidylserine (PS). It then breaks up into membrane-enclosed

fragments, known as apoptotic bodies, which are recognized and engulfed rapidly by neighbouring cells or macrophages thereby inhibiting an inflammatory reaction. Finally, during the secondary necrosis, the remaining cellular structures disintegrate while the phagosome fuses with a lysosome, forming a phago-lysosome.

Apoptosis signalling

During apoptosis signalling three different stages can be distinguished, initialization, execution and degradation. In the initialization phase, signal transduction cascades are activated which will start the effector phase. Various stimuli can trigger apoptotic cell death. These stimuli can be divided in two major classes, physiological signals and cytotoxic agents. Cytotoxic agents are genotoxic, DNA damaging factors like drugs, ionizing radiation (Hickman JA and Boyle CC, 1997). Physiological cell death can be induced by receptor mediated stimuli: cell death signals (glucocorticoids, retinoids, Fas ligand, transforming growth factor beta (TGF–β), tumor necrosis factor alpha (TNF-α)), removal of trophic factors (IL-2, growth factors) or combination of antilogous signals. These signals act via different pathways in mammalian cells: the extrinsic, receptor-mediated, the intrinsic, mitochondria-mediated pathway, caspase-2-dependent and the caspase independent pathway (Fig. 2). In the receptor-mediated pathway, ligation of death receptors is followed by the formation of the death-inducible signalling complex (DISC), which results in the activation of pro-caspase-8 (Muzio M. el al., 1996). In type I cells, caspase-8 (FLICE) activates pro-caspase-3, which cleaves target proteins, leading to apoptosis. In type II cells, caspase-8 cleaves Bid, which, in turn, induces the translocation, oligomerization and insertion of Bax and/or Bak into the mitochondrial outer membrane (Schmitz I *et al.,* 1999). This is followed by the release of several proteins from the mitochondrial intermembrane space, including cytochrome *c*, which forms a cytosolic apoptosome complex with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 in the presence of dATP. This results in the activation of pro-caspase-9, which leads to the activation of pro-caspase-3 (Li P *et al.,* 1997). Mitochondria-mediated apoptosis occurs in response to a wide range of death stimuli, including activation of tumor suppressor proteins (such as p53) and oncogenes (such as c-Myc), DNA damage, chemotherapeutic agents, serum starvation, and UV radiation. This cell death pathway is controlled by Bcl-2 family proteins (regulation of cytochrome *c* release), inhibitor of apoptosis proteins (IAPs) (inhibition of caspases), and second mitochondrial activator of caspases (Smac) and Omi (negative regulation of IAPs) (Shi Y 2001). The intrinsic pathway might also operate

through caspase-independent mechanisms, which involve the release from mitochondria and translocation to the nucleus of at least two proteins, apoptosis inducing factor (AIF) and endonuclease G (EndoG). The nuclear location of AIF and EndoG is linked to chromatin condensation and the appearance of high-molecular-mass chromatin fragments (Penninger JM and Kroemer G 2003). In the caspase 2 dependent pathway the activation of pro-caspase-2 by DNA damage leads to the release of cytochrome *c* and apoptosome formation (as described for the intrinsic pathway), although the precise mechanism by which this occurs is unclear (Lassus, P *et al.,* 2002). Recently, a new caspase-independent, granzyme A (GrA)-mediated pathway was described. GraA is a serine protease which after being delivered into the target-cell cytosol through Ca^{2+}-dependent, perforin-mediated pores, GrA triggers a pathway that is characterized by the formation of single-stranded DNA nicks and the appearance of apoptotic morphology (Beresford PJ *et al.,* 2001).

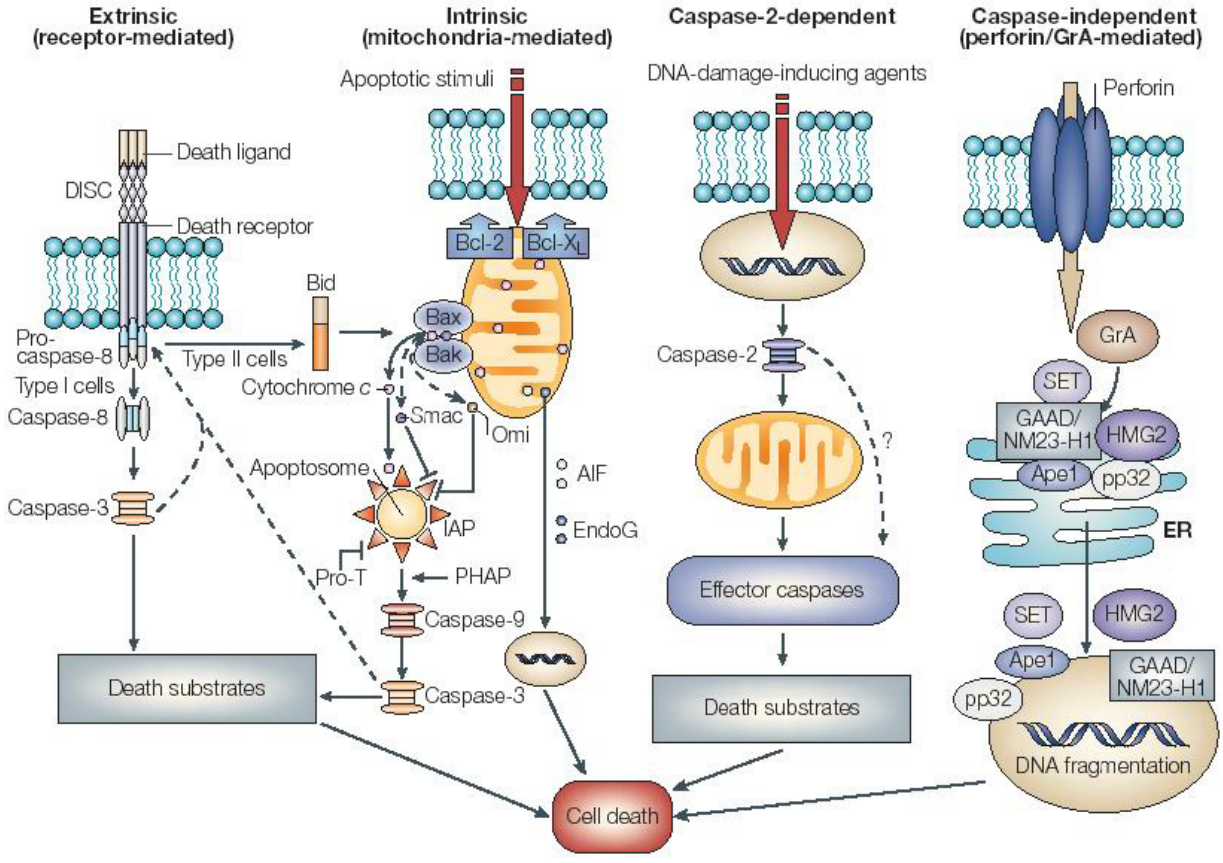

Figure 2 Signalling pathways in apoptosis (based on Orrenius S. *et al.,* 2003)

Activation of the effector caspases leads to the cleavage of several cellular proteins among others Bcl-2, PARP, actin, gelsolin, nuclear lamins and ICAD (Inhibitor of the caspase-activated

DNAse). The effector phase is followed by the degradation phase, which is characterized by the appearance of apoptotic phenotype: DNA fragmentation, zeiosis and finally apoptotic bodies. Beside the morphological changes, a number of changes take places in the cell membrane: loss of phospholipid asymmetry, exposure of new antigens. PS and sugar residues on cell-surface carbohydrates will be recognized by specific receptors (PS receptor, tyrosine kinase receptor MER, scavenger receptor A (SR-A), CD36, CD14, $\alpha_v\beta_3$, $\alpha_v\beta_5$ integrins and lectins) on the macrophages and trigger the ingestion of the apoptotic prey (Savill J, Fadok V, 2000) (Fig. 3).

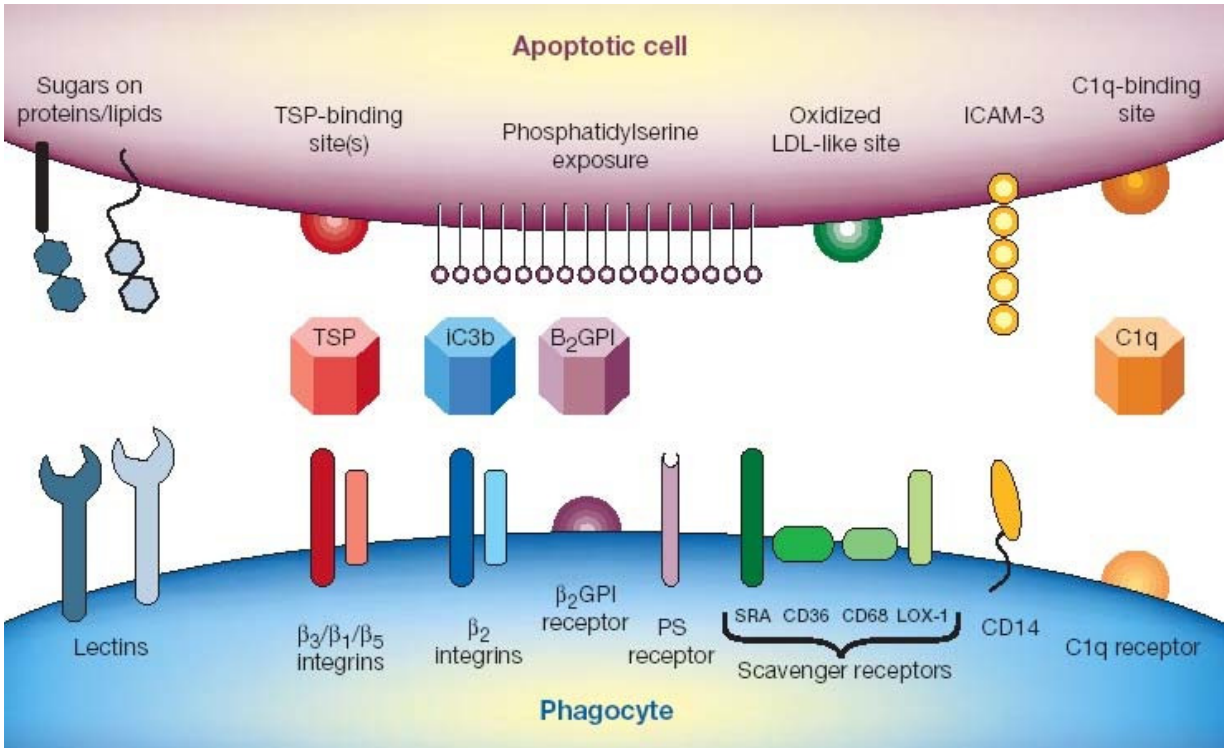

Figure 3 The phagocyte recognition array and its ligands in the mammalian clearance of apoptotic cells. (based on Savill J and Fadok V, 2000)

Mechanism of phagocytosis

All the apoptotic bodies formed are rapidly removed by "amateur" neighbouring cells or "professional" macrophages. Insufficient phagocyte capacity for apoptotic cells can result in failure to clear dying cells before membrane integrity is lost, resulting in leakage of noxious cell contents and severe tissue damage or in case the apoptotic cell is taken up by the antigen presenting dendritic cells, in autoimmunity. Moreover, uptake of apoptotic cells leads to increased production and release of the anti-inflammatory and immunosuppressive TGF-β cytokine actively suppressing the secretion from activated macrophages of pro-inflammatory

mediators such as TNF-α (Fadok VA. *et al.,* 1998a). In contrast phagocytosis of immunoglobulin G-opsonized apoptotic cells results in TNF-α production by macrophages threatening conversion of the anti-inflammatory clearance of dying cells into a pro-inflammatory event.

Transglutaminases

Transglutaminases (TGases; EC 2.3.2.13) (Greenberg CS *et al.,*1991) are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between gamma-carboxiamide groups of peptide-bound glutamine residues and various primary amines, including the epsilon-amino group of lysine in certain proteins. The reaction results in post-translational modification of proteins by establishing N-epsilon-(gamma-glutamyl)-lysine cross-linkages and/or covalent incorporation of polyamines and histamine into proteins. It also has been shown to catalyze esterification of involucrin in keratinocytes (Nemes Z *et al.,* 1999) and to display N-epsilon-(gamma-glutamyl)-lysine isopeptidase (Parameswaran KN *et al.,* 1997) and deaminase (Molberg O. *et al.,* 1998) activities (Fig. 4).

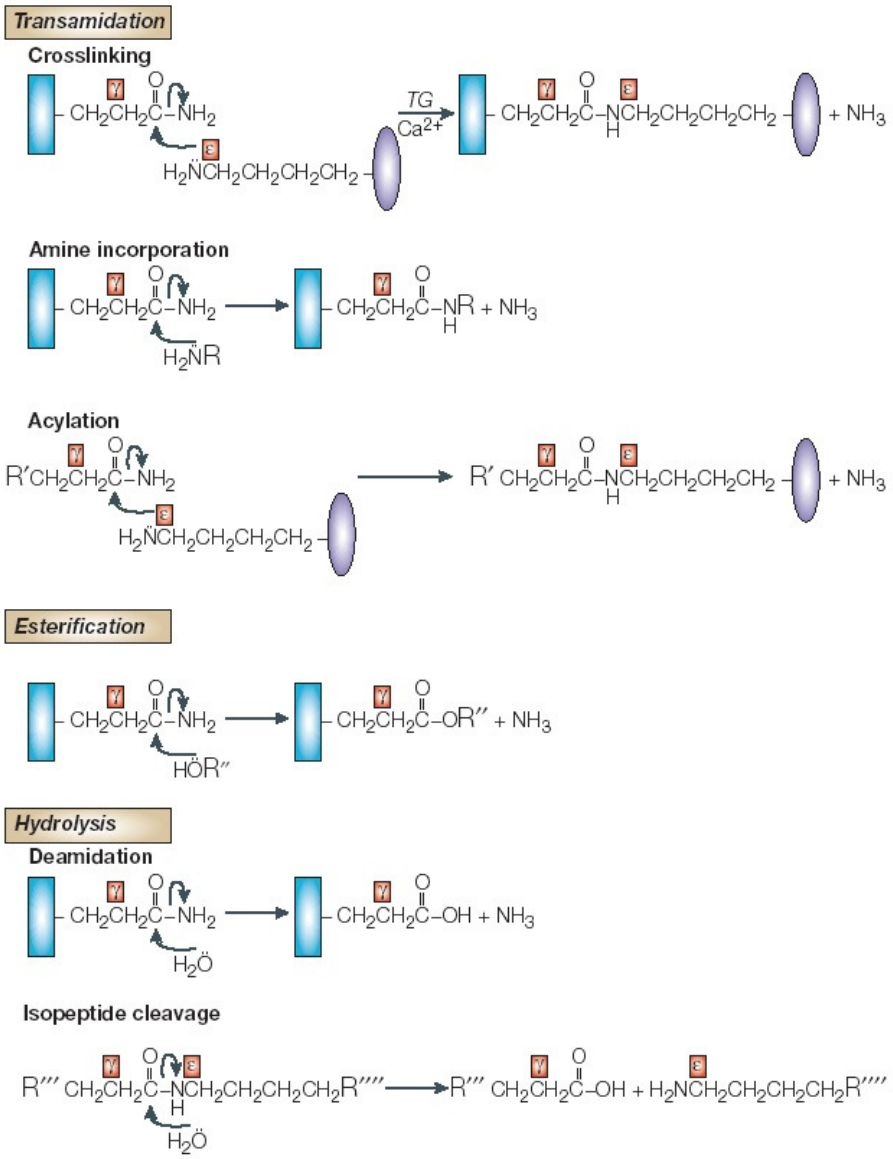

Figure 4 Post-translational modifications catalyzed by transglutaminases (based on Laszlo Lorand and Robert M.Graham 2003).

Based on the presence of the catalytic triad of Cys-His-Asp or Cys-His-Asn eight distinct enzymatically active transglutaminases (Table 1) have so far been described (Grenard, P *et al.,* 2001). Functions include fibrin clot stabilization in haemostasis, semen coagulation, formation of cornified envelopes in keratinization, stabilization of extracellular matrix (ECM) structures and stabilization of apoptotic envelopes in apoptosis. Besides playing a structural role, transglutaminase cross-linking has recently been shown to have a profound effect on cells by

regulating the biological activity of signalling molecules such as TGF-β, interleukin-2, and midkine, as well as by modulating cellmatrix interactions (Aeschlimann, D and Thomazy V, 2000). Factor XIII, is an extracellular enzyme that plays a pivotal role in the cross-linking and stabilization of fibrin during blood coagulation (Takahashi N. *et al.,* 1986). A tetrameric zymogen, Factor XIII is the last enzyme to be activated (by thrombin) in the coagulation process. Another TGase (TGase4) that also exists as a multimeric protein has been isolated from rat prostate. A 150-kDa protein consisting of two identical 71 kDa monomers, $TGase_p$ is a major secretory product of rat dorsal prostate and coagulating gland (Ho KC *et al.,* 1992). Except for Factor XIII and $TGase_p$, all other TGases exist as monomeric single chain proteins. Four of the eight human TGases (1, 2, 3, 5) are expressed in terminally differentiating epithelia. Keratinocyte (TGase1) and epidermal (TGase3) TGases are the major enzymes involved in the formation of CEs during terminal differentiation of epidermis (Peterson LL *et al.,* 1983). TGase5, previously

Protein	Synonyms	Residues (*Mr*, kDa)	Tissue expression	Localization	Function	Gene	Gene map locus	Inherited disease
fXIII subunit A (fXIIIA)	fibrin-stabilizing factor, Laki-Lorand factor, Pro-fibrinoligase, plasma pro-TG	732 (83)	Platelets, astrocytes, dermal dendritic cells, chondrocytes, placenta, plasma, synovial fluid	Cytosolic, extracellular	Blood coagulation , bone growth	*F13A1*	6p24–25	fXIII deficiency
TG1	TG_K, keratinocyte TG, particulate TG	814 (90)	Keratinocytes, brain	Membrane, cytosolic	Cell-envelope formation	*TGM1*	14q11.2	Lamellar ichthyosis
TG2	TG_C, tissue TG, endothelial TG, erythrocyte TG, Gh, Ghα	686 (80)	Ubiquitous	Cytosolic, nuclear, membrane, cell surface, extracellular	Multiple	*TGM2*	20q11–12	Unknown
TG3	TG_E, epidermal TG, callus TG, hair follicle TG, bovine snout TG	692 (77)	Squamous epithelium, brain	Cytosolic	Cell-envelope formation	*TGM3*	20q11–12	Unknown
TG4	TG_P, prostate TG, vesiculase, dorsal prostate protein 1 (DP1), major androgen-regulated prostate secretory protein	683 (77)	Prostate	Unknown	Semen coagulation in rodents	*TGM4*	3q21–22	Unknown
TG5	TG_X	719 (81)	Ubiquitous except for the CNS and lymphatic system	Unknown	Unknown	*TGM5*	15q15.2	Unknown
TG6	TG_Y	Unknown	Unknown	Unknown	Unknown	*TGM6*	20q11	Unknown
TG7	TG_Z	710 (?)	Ubiquitous	Unknown	Unknown	*TGM7*	15q15.2	Unknown
Band 4.2	B4.2; ATP-binding erythrocyte membrane protein band 4.2	690 (72)	Red blood cells, bone marrow, fetal liver and spleen	Membrane	Membrane skeletal component	*EPB42*	15q15.2	Hereditary spherocytosis

Table 1 Calcium-regulated human transglutaminases and a catalytically inactive homologue (based on Laszlo Lorand and Robert M.Graham 2003)

$TGase_X$, is induced during the early stages of keratinocyte differentiation and cross-links loricrin, involucrin, and small proline-rich proteins *in vitro* (Candi E *et al.,* 2001). The functions of TGase6 ($TGase_Y$) and TGase7 ($TGase_Z$) have not yet been characterized. A major membrane skeletal protein in erythrocytes, band 4.2 is a transglutaminase-like molecule, which lacks the active site cysteine residue and thus is catalytically inactive (Sung LA *et al.,* 1992) Tissue TGase (TGase2), is a ubiquitous multifunctional protein and a bifunctional enzyme with both protein-cross-linking and GTP hydrolysing activities (Lee K. *et al.,* 1989). Despite the intensive investigations over many years, its physiological role remains elusive (Fesus L and Piacentini M. 2002). Despite the lack of a leader sequence or post-translational modifications, TGase2 is found on the cell surface and in the ECM, although its function here might not necessarily be a crosslinking one. In fact, TGase2 contributes significantly to the organization of ECM: first, by mediating cell–matrix interactions that affect cell spreading and migration crucial for wound healing (Akimov SS. and Belkin AM. 2001); second, by promoting tissue mineralization (Wozniak M *et al.,* 2000); third, by stabilizing dermo–epidermal junctions (Raghunath M. *et al.,* 1996). Several basement-membrane components, including the laminin–nidogen complex, osteonectin, fibronectin and collagen type VII are stabilized by TGase2 catalysed crosslinks. TGase2 also has an interesting role in the activation TGF-β in the ECM (Kojima S *et al.,* 1993). TGase2 catalyses the covalent attachment of TGF-β, through its latent TGF-β binding protein (LTBP) subunit, to the microfibrils. As such, large stores of this important growth factor can accumulate in the connective tissue from where it can be liberated by the action of proteases. TGase2 is also transported into the nucleus by an interaction with the nuclear transport protein, importin-α3 (Peng X. *et al.,* 1999). In addition to catalyzing the protein cross-linking reactions, it can also hydrolyze ATP and GTP (Iismaa SE *et al.,* 1997). As a guanosine nucleotide binding protein TGase2 was shown to be identical to a previously characterized atypical, high-molecular weight G-protein, known as $G_{alpha(h)}$. In this role as a G protein, the $G_{alpha(h)}$ molecule is associated with a 50-kDa B subunit (G_{beta}), from which it dissociates following stimulation through the α1-b adrenergic receptor and activates a 69-kDa phospholipase C (PLC-δ1) (Nakaoka H *et al.,* 1994), which in turn produces the two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). These intermediate messengers mediate the activation of protein kinase C (PKC) and increases in intracellular [Ca^{2+}], resulting in calcium oscillations in the cells. The downstream events include cell proliferation. Studies revealed that the N-terminal domain of

TGase2 is required for its TGase activity, whereas its core domain is essential for the hydrolysis of GTP and ATP (Iismaa SE *et al.,* 1997) (Fig. 5).

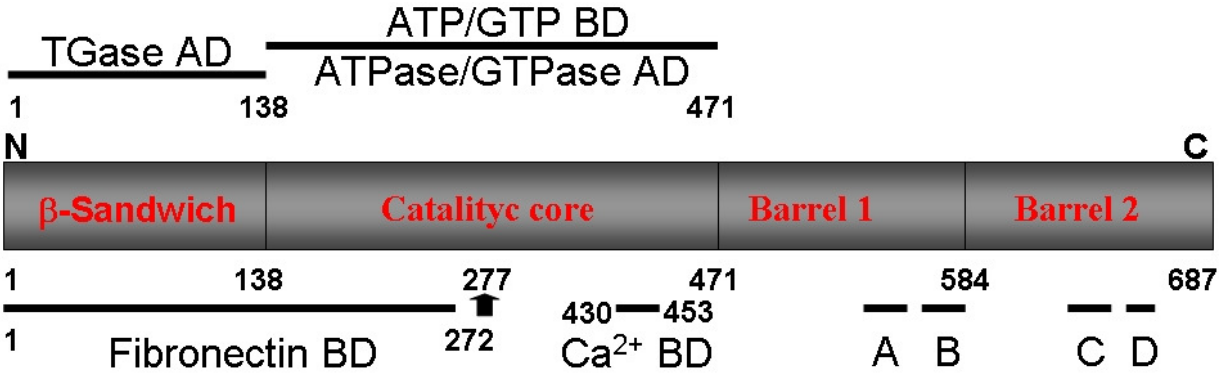

Figure 5 Schematic representation of various binding and functional domains in TGase2 protein. The solid arrowhead denotes the essential cysteine residue in the active site region. Segments A, B, and C denote the three regions, L547-I561, R564-D581, and Q633-E646, respectively, that interact with the α1-adrenoceptor. Segment D denotes the region, V665-K672, critical for interacting and activating PLC. BD: binding domain; AD: activity domain. (based on Chen JS and Mehta K. 1999)

Recently two new functions of TGase2 have been discovered. Hasegawa G and co-workers have shown that TGase2 posses protein disulphide isomerase (PDI) activity which did not require the presence of Ca^{2+} and could be inhibited by bacitracin, a frequently used inhibitor of conventional PDI activity, but not by nucleotides including GTP (Hasegawa G *et al*., 2003). Finally, TGase2 have been proved to have IGFBP-3 kinase activity in breast cancer cells. This kinase activity was inhibited by inhibitors of TGase2 such as cystamine and monodansyl cadaverine, and by increasing concentrations of Ca^{2+} (Mishra S and Murphy LJ, 2004).

The concept that TGase2 actively participates in apoptosis firstly aroused in our department (Fesus L *et al*., 1987). Since then, the role TGase2 in apoptosis has been extensively investigated over the years. It was shown in numerous experimental models that TGase2 is both induced and activated during apoptosis (Fesus L. *et al.,* 1996a). Among others, increased TGase2 activity was measured in apoptotic hepatocytes (Fesus L. *et al.,* 1987) and during the activation, retinoid, glucocorticoid and DNA damage induced apoptosis of T cells (Szondy Z. *et al.,* 1997a; Szondy Z. *et al.,* 1997b). Moreover, N-epsilon-(gamma-glutamyl)-lysine crosslinks have been detected in apoptotic bodies (Fesus L. *et al.,* 1987). In line with this, *in vivo* induction of apoptosis resulted in an increase of the epsilon(gamma-glutamyl)lysine isodipeptide, the end-product of the digestion of transglutaminase cross-linked proteins in the plasma (Szondy Z *et al*., 1997a). In

addition, overexpression of TGase2 promotes apoptosis, whereas its inhibition by antisense constructs protected against cell death (Oliverio S *et al.,* 1999). Other studies have indicated that, by a mechanism thought to involve hyperpolarization of the mitochondrial membrane, TGase2 might act as a sensitizer of death stimuli (Piacentini, M *et al.,* 2002). In other instances, apoptosis has been shown to proceed without the induction of TGase2 expression (Monczak Y *et al.,* 1997). Monodansylcadaverine, which binds to the transamidase active site of TGase2, eliminated retinoic acid (RA) protection against cell death and in fact caused RA to become an apoptotic factor in HL60 cells. This protection was shown due to an interaction between TGase2 and retinoblastoma protein and post-translational modification of Rb (Boehm JE *et al.,* 2002). However, the fact that transamidase activity deficient mutant TGase2 was still able to protect against apoptosis and mutants defective in GTP binding lost this ability indicates a role for TGase2 in more than one anti-apoptotic signalling pathway (Antonyak MA *et al.,* 2001). Therefore, TGase2 might be neither necessary nor sufficient for programmed cell death. However, through its ability to crosslink cytoskeletal, intermediate and nuclear proteins into insoluble aggregates, it could be important for preventing the inflammatory responses that would otherwise follow disruption of dying cells and the dispersion of their contents.

Regulation of TGase2 expression and activity

Analysis of the structure of the promoters of several tissue transglutaminase genes has demonstrated that the core promoters, which contain TATA-, CAAT-box motifs, series of transcription factor-binding sites (retinoid, AP1, SP1, IL-6, glucocorticoid response elements) and CpG-rich flanking region, show a high basal transcriptional activity in many types of cells. Even though the core tissue transglutaminase promoter is constitutively active (Lu S *et al.,* 1995), the intact transglutaminase gene is expressed in a highly regulated manner and may be under negative regulatory control. Several factors are known to induce TGase2 expression, e.g.: cAMP, glucocorticoid, IL-6, RA, TGF-β, TNF-α. Latent TGase activities can be turned on inside cells in the absence of protein synthesis by increasing the concentration of intracellular Ca^{2+}, which is consistent with the situation that occurs *in vitro*. Given the relatively high concentration of Ca^{2+} that is required for the activation of TGases, it can further be assumed that TGase activation occurs mainly during extreme situations when the internal Ca^{2+} storage capacity of the cell becomes overwhelmed, or when the outward directed Ca^{2+}-pumps fail to keep up with influx of the cation. Guanosine nucleotides can also modulate the protein cross-linking activity of TGase2.

In particular, it was found that GTP binds noncompetitively to purified guinea pig TGase2 and inhibits its Ca^{2+}-dependent cross-linking activity (Achyuthan KE and Greenberg CS 1987). The binding of GTP causes a conformational change that reduces the affinity of TGase2 for Ca^{2+} (Bergamini CM 1988). Addition of Ca^{2+}, on the other hand, reverses the inhibitory effect of GTP, suggesting that the respective Ca^{2+} and GTP concentrations may help regulate the two catalytic functions of TGase2.

Apoptosis in the thymus

Immature thymocytes begin life as emigrants from the bone marrow. When they arrive in the thymus, these precursors do not express CD4, CD8, or T cell receptor (TCR). Once the mature TCR is expressed, the TCR^{+} double positive thymocytes undergo two selective steps. During the T-cell selection, cells bearing TCRs, which are unable to bind to self-MHC molecules, die in the cortex (death by neglect). In negative selection, autoreactive clones bearing TCR which have a "high" affinity for self-antigen are deleted, i.e. induced to undergo apoptosis. The appropriate, intermediate level of TCR signalling initiates effective differentiation (positive selection) of T cells. Apoptosis can be induced by various signalling pathways in immature T cells: glucocorticoids act through glucocorticoid receptors (Gruol DJ and Altschmied J 1993), retinoids through RARγ (Szondy Z *et al.,* 1997b), TCR activation during the negative selection through the transcription factor nur77 (Woronicz JD *et al.,* 1994) ionizing radiation via the activation of p53 (Lowe SW *et al.,* 1993) and FasL (Owen-Schaub LB *et al.,* 1992). Except the latter one, which directly activates caspases, all signal pathways require *de novo* protein synthesis. Besides the RARγ-mediated apoptosis inducing effect, RA also play important role in regulation of negative selection and T cell differentiation. Studies in our laboratory have demonstrated that RARα ligation selectively inhibit TCR-mediated death (Szondy Z *et al.,* 1998; Szegezdi E *et al*., 2003). These cells not only escape negative selection but also further differentiate to mature, peripheral T cells, suggesting that RAs are capable to affect the threshold of the negative selection. (our, yet unpublished observation).

Apoptosis in the liver

Apoptosis in the liver has become the focus of many researchers since it became apparent that deregulation of the apoptotic program is pathophysiologically involved in liver diseases such as alcoholic liver disease, viral hepatitis, hepatocellular carcinoma and cholestatic liver diseases.

The Fas signalling has been proposed as an important pathogenic mechanism in all these diseases (Rust C and Gores GJ, 2000). After triggering of the Fas by FasL or agonistic antibodies, the receptors trimerize and the intracellular "death domain" (DD) of the receptor recruits two DD containing proteins: FADD and Rip. In addition to its DD, FADD also contains a so-called death effector domain (DED), which recruits caspase-8. According to the „induced proximity model", oligomerization of the recruited pro-caspase-8 is sufficient for its autoproteolytic activation, upon which the active subunits of caspase-8 are released into the cytoplasm. This leads to a cascade of caspase activation events induced by caspase-8 (Sartorius U *et al*., 2001). FLICE-inhibitory protein (FLIP), a catalytically inactive caspase-8/-10 homologue, can interrupt the Fas-triggered apoptotic pathway by binding the FADD, thus blocking the association between the Fas/FADD complex and caspase-8, the earliest caspase in the pro-apoptotic cascade initiated by Fas engagement (Irmler M *et al*., 1997). In addition to other caspases caspase-8 can cleave Bid, which induces the translocation, oligomerization and insertion of Bax and/or Bak into the mitochondrial outer membrane (Schmitz I. *et al.,* 1999). This is followed by the release of several proteins from the mitochondrial intermembrane space, including cytochrome *c*, which forms a cytosolic apoptosome complex with Apaf-1 and pro-caspase-9 in the presence of dATP. This results in the activation of pro-caspase-9, which leads to the activation of pro-caspase-3 (Li P *et al.,* 1997). Activation of Fas-mediated apoptosis *in vivo* can be achieved by a single injection of an agonist anti-Fas antibody. Mice usually die within 6 h of receiving a lethal dose of anti-Fas antibody, and $Fas^{lpr/lpr}$ mice that do not have functional Fas are completely resistant to this treatment. The major organ affected is the liver and the massive hepatocyte apoptosis is responsible for the animal death (Ogasawara J *et al*., 1993). Caspase-3/7/8 activities are detected in liver cells of mice injected with anti-Fas antibodies as well as in hepatocytes incubated with anti-Fas *in vitro* (Kunstle G *et al*., 1997). Besides caspase inhibitors, apoptosis can be inhibited by transgenically overexpressing Bcl-2 or Bcl-x_L (de la Coste A *et al*., 1999) suggesting that the mitochondrial pathway is activated by the Fas signalling in hepatocytes. On the other hand, after partial hepatectomy, which triggers immediate liver regeneration, Fas engagement was reported to accelerate liver regeneration possibly as a result of maintaining the intracellular level of the long form of FLIP (Desbarats J and Newell MK, 2000). Besides hepatocytes, hepatic sinusoidal endothelial cells are also susceptible to Fas-mediated apoptosis further contributing to the Fas-induced hepatic failure (Wanner GA *et al*., 1999).

Role of TGase2 in the proliferation and death of hepatocytes

In the liver, both parenchymal and non-parenchyma cells produce TGase2, and TGase2 appears to be released into the extracellular space. Due to its cross-linking and GTPase activities, it has been shown to participate in both the apoptosis and in the proliferation of hepatocytes, respectively. Treatment of cultured rat hepatocytes with ethanol led to apoptosis, an increase in cytosolic cytochrome C levels, and enhanced TGase2 activity in cytosol and nuclear compartments (Song LW *et al.,* 2001). These findings suggest the concept that TGase2 is a crucial factor in the apoptotic process caused by a variety of factors. It is well established that ethanol exposure inhibits hepatocyte mitogenesis and proliferation (Wands JR. *et al.,* 1979). Ethanol has been shown to decrease putrescine levels in the liver, and the administration of putrescine or its precursors, alanine or glutamine, abrogates this inhibition of liver regeneration by ethanol administration (Diehl AM. *et al.,* 1990). The mechanism by which putrescine abolishes this effect of ethanol on regeneration may be that putrescine acts as a competitive substrate inhibitor of TGase2 activity. It has been demonstrated that α_1-adrenergic agonists such as epinephrine and phenylephrine enhance hepatocyte proliferation (Cruise JL. *et al.,* 1985). A variety of hormones and other agonists act through specific receptors coupled to different G proteins to stimulate distinct classes of PLC isozymes that are all capable to rise the intracellular [Ca^{2+}] level and subsequent to induce proliferation. However, previous studies established that the enhancement of cell proliferation by G protein-coupled receptor agonists is much more potent for phenylephrine than for other hormones acting through different receptors (Houck KA and Michalopoulos GK, 1989). Moreover, the data of Wu J. and co-workers implies that the $G_{alpha(h)}$-dependent activation of PLC-δ1 by phenylephrine is preferentially involved in the enhancement of cell proliferation (Wu J *et al*., 2000).

Aim of the study

The overall aim of the first part of the present study was to investigate the apoptotic program in the absence of TGase2 with emphasis on thymic and hepatic cell death.

The specific sub-aims were to:

- determine if there are differences in the *in vivo* and *in vitro* apoptosis of thymocytes from WT and TGase2$^{-/-}$ mice
- determine whether loss of TGase2 is compensated by other TGases
- determine if the lack of TGase2 in the knock-out mice effects the clearance of apoptotic cells
- investigate if TGase2 is required for proper corpse clearance in the thymocytes or in the macrophages
- identify possible factors which are required for proper phagocytosis in WT mice and are absent, or present in lower amount in TGase2$^{-/-}$ mice
- investigate the long-term effects of the TGase2 deficiency
- compare *in vivo* and *in vitro* sensitiveness of WT and TGase2$^{-/-}$ hepatocytes to Fas-mediated apoptosis
- characterize cell death induced by anti-Fas antibody in the livers of WT and TGase2$^{-/-}$ mice
- explain the preventive role of TGase2 on Fas-mediated hepatic injury

Materials and Methods

Animals. TGase2$^{-/-}$ mice lacking of part of exon 5, intron 5, exon 6, and a piece of intron 6 of the transglutaminase 2 gene (De Laurenzi V, Melino G, 2001) and their wild-type counterparts were obtained from Gerry Melino's laboratory. α1β-adrenoceptor knock-out mice lacking of exon 1 of the $\alpha_1-\beta$ adrenoceptor gene (Cavalli A *et al*., 1997) and WT mice were obtained from Susanna Cotecchia's laboratory. Study protocols were approved by the Animal Care Committee of the University of Debrecen.

Induction of thymic apoptosis in vivo. To induce thymic apoptosis four weeks old TGase2$^{+/+}$ and TGase2$^{-/-}$ mice were injected with 50 μg anti-CD3 mAb (Pharmingen), 0.5 mg dexamethasone-acetate (DXM) (Sigma), exposed to 5 Gy of γ-irradiation or 1μg/g body weight Jo2 anti-CD95 mAb (Pharmingen). In some experiments 25 μg neutralizing anti-TGF-β mAb (R&D Systems) was injected 12 h prior to the treatment and a second dose at the time of apoptosis induction. Controls received the same dose of isotype control mAb (Pharmingen). Thymic apoptosis was evaluated 24 hrs later by measuring the change in amount of thymic weight and analyzing cellular changes. For the latter, thymocytes were isolated, washed twice and resuspended in ice-cold phosphate buffered saline (PBS) before staining either with PE labelled anti-CD4 and FITC-conjugated anti-CD8 (Pharmingen) antibodies or with FITC-labelled annexin-V in binding buffer (Sigma). Cell-bound fluorescence was analyzed in a blinded fashion using a FACScalibur (Beckton Dickinson). Since the total cellularity of thymus in wild-type and TGase2$^{-/-}$ mice was different, each data obtained following apoptosis induction was expressed as percentage of the non-treated littermates.

Thymocyte apoptosis in vitro. Thymocyte suspensions were prepared by mincing thymus of four weeks old mice in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 1mM Na-pyruvate, 5 x 10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. After washing thymocytes were diluted to a concentration of 10^6 cells/ml and cultured at 37^{o}C / 5% CO_2. Apoptosis was induced by addition of 0.1 μM DXM, 10 μg/ml anti-CD3 mAb, 5 μM etoposide (Sigma) or 1 μg/ml anti-CD95 mAb. In some experiments 5 ng/ml TGF-β1 (R&D System) was also added. After 6 hrs, the extent of cell death was determined by 7-aminoactinomycin D uptake (Lecoeur H *et al.,* 1998).

Induction of hepatocyte apoptosis in vivo was carried out at Gerry Melino's laboratory in Rome. Hepatocyte apoptosis was induced by intravenous injection of $PbNO_3$ [10μmol/100g (Carlo Erba, Milano, Italy)] dissolved in physiological saline. For histological examination and evaluation of the incidence of apoptosis, small fragments of tissue were formalin-fixed, embedded in paraffin and stained with haematoxylin-eosin. The percentage of apoptotic cells was assessed by light microscopy i. e. counting of 500-600 hepatocytes per sample on 5 different sections.

Determination of TGase activity. Thymus was collected, extensively washed with PBS, and homogenized in 0.1 M Tris·HCl, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Transglutaminase activity was measured by detecting the incorporation of [^{3}H]putrescine into N,N-dimethylcasein. The incubation mixture contained 150 mM Tris·HCl buffer, pH 8.3, 5 mM CaCl2, 10 mM dithiothreitol, 30 mM NaCl, 2.5 mg N,N-dimethylcasein/ml, and 0.2 mM putrescine, containing 1 mCi of [^{3}H]putrescine and 0.1 mg of protein in a final volume of 0.3 ml. After 30 min of incubation, the mixture was spotted onto Whatman 3 MM filter paper moistened with 20% trichloroacetic acid. Free [^{3}H]putrescine was eliminated by washing with large volumes of cold 5% trichloroacetic acid containing 0.2 M KCl before counting. Activity was calculated as nmol of [^{3}H]putrescine incorporated into protein/hr.

Western blot analysis of TGase2 enzyme. Samples from thymus tissue homogenates containing 1 mg/ml protein were mixed with an equal volume of sample buffer (0.125 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerin, 10% mercaptoethanol and 0.02% bromphenol blue). Electrophoresis was performed in a 10% SDS-polyacrylamide gel. Separated proteins were electroblotted and probed with monoclonal anti-TGase2 (CUB7402), polyclonal anti-TGase2 (Upstate Biotechnology) antibodies to TGase2. Bound antibodies were visualized using the Amersham ECL detection system.

Determination of the expression of TGase1,2,3,5,7 using quantitative PCR. Real-time quantitative PCR was carried out on an ABI PRISM 7700 Sequence Detection System using the 5' nuclease assay at Daniel Aeschlimann's laboratory in Wales. Total RNA was isolated from thymus 12hrs after induction of apoptosis, and 4 μg of it was reverse transcribed into DNA using 100units SuperScript II reverse transcriptase (Life Technologies, Inc) and 0.025μg/μl oligo(dT)15 primer. PCRs were carried out using the primers and concentrations indicated below.

Quantitative PCR design.

	Forward primer	Concentration [nM]
TGase1	CTCGGACTCTGTGACCATGC	150
TGase2	CTCGGACTCTGTGACCATGC	150
TGase3	GCAGCCTCAAGATTGATGTGC	600
TGase5	AGCAGCAGAGAGTTCTCATTGG	300
TGase7	GGCAGATGACAAAAGACCTTGG	300
Ribosomal protein-S26	AGATGCAGCAGATCCGCAT	300
	TaqMan probe (Applied Biosystems)	Concentration [nM]
TGase1	TGCTCAATGTCTCAGGCCACGTCAAGG	150
TGase2	AGCTACCTGCTGGCTGAGAGAGATCTC	175
TGase3	CGGAGTGGCACCAAGCAACTGCTCGC	125
TGase5	AGACCGTCCCCTTCAAGAGTGGCCAAAGG	150
TGase7	CTCTACCCCATCAAAGCTGGGCCCCAC	150
Ribosomal protein-S26	AGGCTGTGGTGCTGATGGGCAAGAAC	100
	Reverse primer	Concentration [nM]
TGase1	CAACTGCTGCTCCCAGTAACG	150
TGase2	CAGTTTGCGGTTTTGCTTGG	150
TGase3	AGACATCAATGGGCAGCATGG	600
TGase5	CCTTGATGTCTTTAAACCTATTGCTC	300
TGase7	CCTTGACCTCACTGCTGCTGA	600
Ribosomal protein-S26	ATATGAGGCAGCAGTTTCTCCAG	300

Calculation of starting concentration was based on standard curves for target DNA run in parallel. Ribosomal protein S26 was used as an internal reference of housekeeping gene transcription for normalization between different cDNA samples.

Transmission electron microscopy was carried out at the Department of Pathology. For transmission electron microscopy analysis thymus samples and livers were fixed with 1% glutaraldehyde in 0.1M cacodilate buffer, cut in small pieces and postfixed with 2% OsO_4 in the same buffer. Tissue samples were then dehydrated and embedded in epoxy resin (Spurr). Ultrathin sections were stained with uranyl acetate and lead nitrate and observed under a Zeiss EM900 electron microscope.

Macrophage phagocytosis assays. Macrophages were harvested from adult mice by peritoneal lavage with RPMI 1640 medium and seeded on 2% gelatine treated 24-well plates in RPMI 1640 medium containing 10% FCS (2×10^5 cells). After 2 hrs, nonadherent cells were washed away. Thymocytes were isolated from 4 weeks old mice. Opsonized thymocytes were prepared by

incubation with anti-CD3 or isotype-matched antibody (rat IgG2b, Pharmingen, 1 μg per 10^6 cells). For generation of apoptotic cells, thymocytes were induced to die by addition of 0.5 μM calcium ionophore A23187 for 6 hrs. After rinsing, each type of cell preparation was placed on top of the macrophages at a concentration of 10^7 cells/ml in RPMI 1640 medium containing 10 % FCS. After 1 h of co-culture thymocytes that had not been taken up were washed away, while the adherent cells were prefixed with 0.5 % paraformaldehyde in 0.1 M Sörensen phosphate (SP) buffer (pH 7.4) for 30 min, and fixed with 2.5 % glutaraldehyde in SP buffer for 3hrs at room temperature. After extensive rinsing cells were „stained" with 2% osmium tetraoxide for 30 min at room temperature for contrast enhancement and studied by phase contrast microscopy. For visualising *Listeria monocytogenes* uptake, bacteria were fluorescein-labelled with 2 μM CellTracker green (Molecular Probes) in Luria broth media (Difco) for 30 min, and then washed in RPMI 1640 medium, before addition to macrophage cultures. Cells were fixed with 1% paraformaldehyde and analyzed using epifluorescence microscopy. For detecting the uptake of *S. cerevisiae* the technique described by Simpson and co-workers was applied (Simpson DW *et al.,* 1979).

Autoantibody detection. Serum samples diluted 1:40 with PBS were applied for 30 min on rat liver-kidney–stomach sections purchased from BioSystems SA, Spain. Bound antibodies were detected with FITC-labelled goat anti-mouse IgG (Fc specific) (Sigma). Liver-kidney-stomach sections are routinely used for autoantibody screening in human diseases and enable the detection of antinuclear, anti-smooth muscle, anti-mitochondrial, anti-parietal cell and liver-kidney microsomal antibodies.

Detection of rheumatoid factor type autoantibodies. Autoantibodies specific for mouse IgG isotypes (IgG1, IgG3, IgG2a and IgG2b) were detected by solid phase enzyme-linked immunosorbent assay (ELISA) as described previously (Fazekas G *et al.,*1995). Microtiter plates were coated with pretitrated amounts (2.5 - 5μg/ml) of IgG and incubated with 1:20, 1:100, 1:500 and 1:2500 dilutions of sera. Bound antibodies were detected with the mixture of isotype-specific biotinylated rabbit antibodies (lacking the reagent for the coating isotype) (Amersham). Results are given as the mean absorbance at 492nm + SD of the 1:100 serum dilution.

Detection of IgM-containing immune complexes. Kidney was removed, horizontally sliced and tissue blocks snap-frozen with isopentane in liquid nitrogen. 4μm cryosections were cut,

fixed in cold aceton, and incubated with FITC-labelled anti-mouse IgM (Sigma), diluted in PBS (1:40). After rinsing in PBS, slides were mounted in buffered glycerol.

Determination of serum urea concentration. The method of Rahmatullah and Boyde was used (Rahmatullah M and Boyde TR, 1980). Briefly, samples were mixed with color reagent (freshly made mixture of diacetyl monoxime, thiosemicarbazide, sulphuric acid and phosphoric acid) in 1:3 ratio and incubated at 85 ^{o}C for 30 minutes followed by incubation at 12 ^{o}C for 10 minutes. Absorbance was measured at 540nm

In vivo anti-Fas treatment of animals. 4 weeks-old, TGase2$^{+/+}$, TGase2$^{-/-}$, ADR$^{+/+}$ and ADR$^{-/-}$ mice received a single intraperitoneal injection of 1 μg/g or 0.3 μg/g body weight of Jo2 anti-Fas antibody. In some experiments TGase2$^{+/+}$ mice were treated for a week with a daily dose of chloroethylclonidine (Sigma), an adrenoceptor antagonist (Michel MC *et al*., 1993), to detect changes in the Bcl-x_L expression and in the *in vitro* Fas sensitivity of hepatocytes, or to follow the pre-treatment by anti-Fas antibody injection.

Hepatocyte culture experiments. Hepatocytes were isolated from wild-type and TGase2$^{-/-}$ mice by the collagenase perfusion method at the Department of Medical Chemistry, SOTE (Banhegyi G *et al*., 1991) and suspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. After washing hepatocytes were diluted to a concentration of 10^6 cells/ml and cultured at 37^{o}C/5% CO_2 for 24 hrs in the presence or absence of increasing concentrations of the anti-Fas antibodies. The percentage of apoptotic cells was determined by counting the percentage of annexin-V-FITC positive and propidium iodide negative cells using the kit provided by Sigma.

Determination of cell surface expression of Fas on hepatocytes of wild-type and TGase2$^{-/-}$ mice. Freshly isolated hepatocytes were washed twice with PBS supplemented with 1%BSA and 0.1% sodium azide (Buffer A). 10^6 cells were resuspended in 100 μl of Buffer A containing 10 μg of anti mouse-FcγR Ab (IgG) and incubated for 15 min at room temperature to saturate nonspecific binding sites. For detection of cell surface Fas, cells were stained with a PE-labelled anti-Fas antibody (Pharmingen). Fluorescence was analyzed on an Epix Coulter FACScan with excitation of the incident light at 488 nm.

Determination of Bcl-x_L and cFLIP(L) expression in the liver of TGase2$^{+/+}$, TGase2$^{-/-}$, ADR$^{+/+}$ and ADR$^{-/-}$ mice. For detection Bcl-x_L and cFLIP(L) protein expressions, cells were

washed once with ice-cold PBS and lysed in hypotonic lysis buffer (20 mM Tris pH 7.5, containing 0.25 M sucrose, 10 mM ditothreitol, 2mM EDTA, 2 mM EGTA, 100 μg/ml aprotinin, 100 μg/ml leupeptin and 1 mM phenymethyl-sulphonyl-fluoride) and then centrifuged for 5 min at 500g. The supernatant was dissolved in an equal amount of sample buffer (0.125 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerin, 10% mercaptoethanol and 0.02% bromphenol blue) and subsequently incubated at 100^{o}C for 10 min. The 10% SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (Laemmli UK, 1970). The separated proteins (equal to 10^6 cells) were electroblotted onto a polyvinyl difluoride membrane. For staining the blot, anti-mouse Bcl-x_L (Transduction Lab) and FLIP (Alexis Biochemicals) antibodies were added, followed by incubation with horseradish peroxidase-labelled, affinity-purified goat anti-rabbit and anti-rat IgG, respectively. Bands were visualized by the Amersham ECL technique. Equal loading of protein was demonstrated by probing the membranes with an anti-actin antibody (Sigma).

Histological examinations of liver were carried out at the Department of Pathology. Livers were dissected under anesthesia, both from anti-Fas antibody injected animals and from saline treated control littermates. Representative blocks of tissue were formalin-fixed, embedded in paraffin and stained with hematoxylin-eosin. For transmission electron microscopy (TEM) analysis livers were fixed with a modified Karnovsky fixative [2% glutaraldehyde + 4% buffered formalin (0.1 M phosphate buffer)] for 2 hrs, followed by osmication (2% OsO_4 for 2 hrs). Tissue samples were then dehydrated and embedded in Araldit resin. Ultrathin sections were "stained" with uranyl acetate and lead citrate and observed under a JEOL 1010 transmission electron microscope.

Estimation of the in vivo rate of apoptosis following anti-Fas injection in the liver. Livers from anti-Fas antibody-treated mice were carefully removed, washed with physiological saline, formalin fixed, embedded in paraffin and stained with the APOPTAG in situ apoptosis detection kit (Intergen Discovery) following the manufacturer's instructions at the Department of Dermatology and Venerology.

Determination of serum AST and ALT levels. To determine the extent of liver injury following anti-Fas antibody injection, serum was collected, in the case of TGase2$^{-/-}$ and ADR$^{-/-}$ mice, at the time of their death, whilst in the case of wild-type mice, at the time points where the knock-outs died. AST and ALT levels were determined by routine clinical chemical methods.

Results

Cell death in the absence of TGase2 in the thymus

Defective clearance of apoptotic cells in the thymus of TGase2$^{-/-}$ mice. Induction of thymic apoptosis by anti-CD3 mAb, DXM or γ–irradiation, signals known to initiate thymocyte death via different signalling pathways, also induced the *in vivo* expression of TGase2 mRNA (up to 700 fold) with a concomitant increase in the enzyme protein and activity in the WT animals (Fig. 7A-C). TGase2 mRNA lacking exons 5 and 6 was expressed in TGase2 $^{-/-}$ mice, but the respective protein was not detectable by immunoblotting with either CUB7402 monoclonal (Fig. 7C) or with polyclonal antibodies (data not shown) suggesting that the aberrant protein is rapidly degraded. Though the basal steady-state level of TGase2 message was higher in the knock-out animals than in the WT controls, the increase observed in knock-out mice after the induction of apoptosis was less than that in the WT animals suggesting that its transcriptional activation is TGase2-dependent (Fig. 7A). Similar results were obtained with other TGases. TGase 1, 3, 5, and 7 were also found to be expressed and induced during the thymic apoptosis program. However in TGase2$^{-/-}$ mice their induction was impaired (Fig. 6).

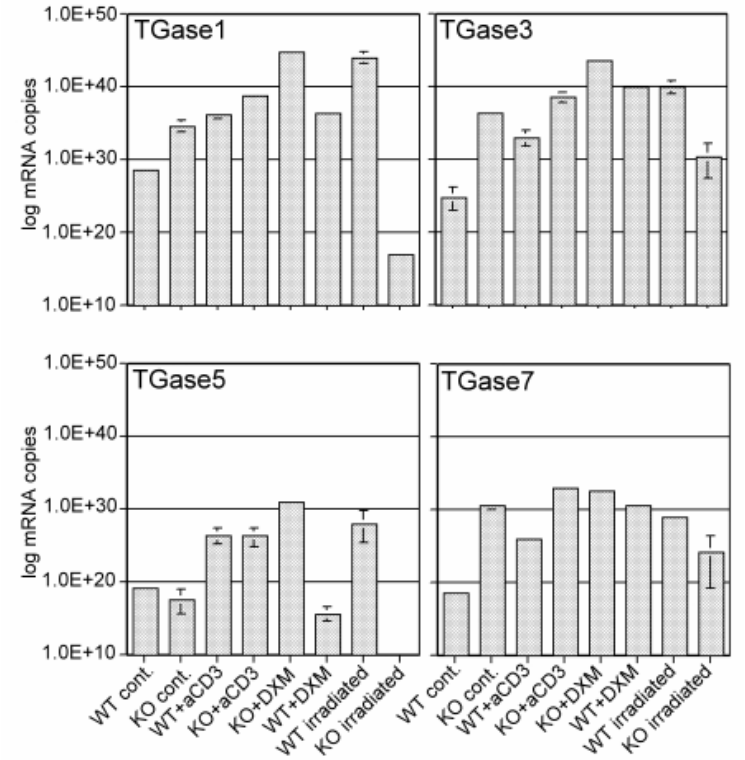

Figure 6 Changes in the expression levels of different TGases mRNAs at 24 h following a single dose of anti-CD3 mAb (50 μg), DXM (0.5 mg) injection or γ–irradiation (5Gy).determined by real-time quantitative PCR.

The thymus in knock-out mice at the age of four weeks was smaller than in WT with a significantly ($p<0.002$) reduced number of thymic cells ($26.2\pm2.3x10^7$ versus $15.9\pm1.9x10^7$, representing mean ± SD for 10 animals), but with no alteration in the percentage of the various thymocyte populations. When the tissue loss in response to apoptotic stimuli was compared between WT and knock-out mice, the percentage loss in thymic weight (Fig. 7D) and cellularity (Fig. 7E) was found to be diminished in the knock-out animals in each treatment group. As all treatments affected only the $CD4^+CD8^+$ population, this difference was most evident when the percentage of the $CD4^+CD8^+$ cells that survived was compared (Fig. 7F). The observed reduced loss of thymic cells in the $TGase2^{-/-}$ mice may be the consequence of either a reduced rate of apoptosis or phagocytosis. To discriminate between these alternatives, the changes in the percentage of the annexin V^+ (free apoptotic) thymocytes following apoptosis induction were also determined. As shown in Figure 7G, thymi of the $TGase2^{-/-}$ mice when compared to WT showed a significant increase in annexin V^+ apoptotic cells. In accordance, electron microscopic analysis of the thymus from WT mice treated with DXM showed typical atrophy of the thymus, with condensed nuclei of apoptotic cells mostly being associated with macrophages (Fig. 7H). In contrast, in the thymus of the $TGase2^{-/-}$ mice free apoptotic bodies are present in abundance, often forming large clusters (Fig. 7I).

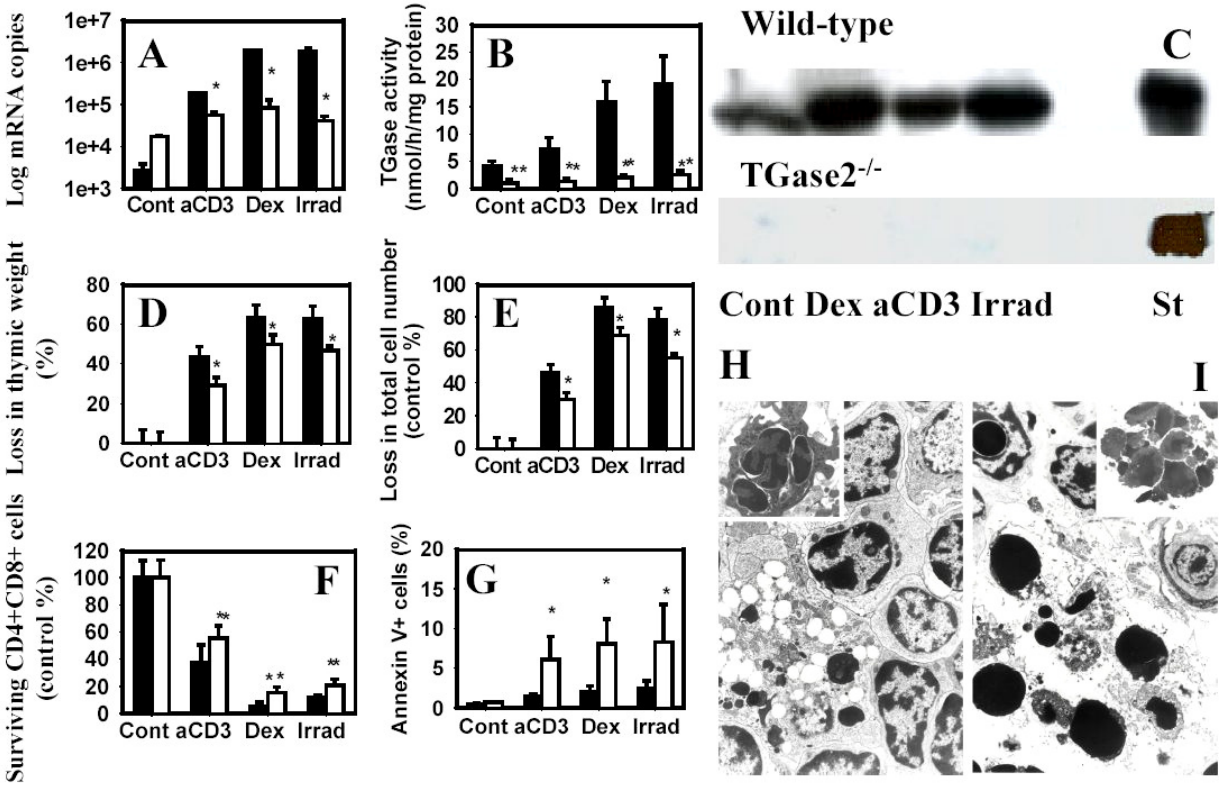

Figure 7 The *in vivo* thymic apoptotic program of TGase2$^{+/+}$ (closed bars) and TGase2$^{-/-}$ (open bars) mice (n=10) at 24 h following a single dose of anti-CD3 mAb (50 μg), DXM (0.5 mg) injection or γ–irradiation (5Gy). (A) Changes in the expression levels of TGase2mRNA determined by real-time quantitative PCR. (B) Changes in the transglutaminase activity expressed as nmoles of [^{3}H]putrescine incorporated per mg protein and per hour. (C) Protein levels of TGase2 detected by Western blot analysis using monoclonal antibody CUB7402. Liver extract from wild-type mice known to express TGase2 was used as positive control. (D) Loss in thymic weight. (E) Loss in total cell number. (F) Survival of CD4$^+$CD8$^+$ thymocytes expressed as percentage of the absolute CD4$^+$CD8$^+$ cell number of non-treated controls, and (G) percentage of annexin-V positive cells of all thymic cells determined by FACS analysis. Data represent mean ±SD. Statistically different from the wild-type mice (*$p<0.05$, **$p<0.004$). (H and I) Electron-microscopic sections of thymi from TGase2$^{+/+}$ and TGase2$^{-/-}$ mice treated with DXM. In wild-type mice (H) apoptotic cells that appear as large electron-dense bodies are found within the macrophages. Insert: one macrophage with three engulfed apoptotic bodies. In TGase2$^{-/-}$ mice (I) an accumulation of free apoptotic bodies is apparent and, as shown in insert, clusters of apoptotic bodies are frequently observed. Original magnification:1,500 X.

Defective clearance of apoptotic cells is accompanied with inflammatory reaction in the liver of TGase2$^{-/-}$ mice. To investigate the clearance of apoptotic cells in a different organ, we injected adult TGase2$^{-/-}$ mice with a single dose of $PbNO_3$. This induces a proliferative response leading to doubling of the liver mass in three days followed by an involution phase that involves

apoptosis and induction of TGase2 protein and activity (Fesus L *et al.,* 1989) and then the liver recovers its weight within 2-3 days. Apoptosis was determined by counting morphologically typical apoptotic cells on tissue sections (Fig. 8B and C). As shown in Figure 8A, in the liver of TGase2$^{-/-}$ mice, at 5 and 10 days post injection, a significant increase in apoptotic cells was detected as compared to wild-type. Concomitant with the accumulation of apoptotic cells, an increase in total liver weight in TGase2$^{-/-}$ mice as compared to WT animals was observed (30% at day 5 and 80% at day 10 post-injection). This delayed recovery of liver weight was characterized by the accumulation of morphologically typical apoptotic cells, mostly not associated with macrophages (Fig. 8E). In contrast, WT mice treated with a similar dose of $PbNO_3$ showed a lower number of apoptotic cells, mostly taken up by Kuppfer cells (Fig. 8F). Even in vehicle-treated animals, a lower amount of apoptotic cells was observed in WT liver as compared to TGase2$^{-/-}$ liver (Fig. 8A). Taken together, these data suggest that TGase2$^{-/-}$ mice have a constitutive deficiency in the clearance of apoptotic cells. Following apoptosis induction, this was associated with an inflammatory response as evidenced by the presence of large infiltrates of blood cells in many parts of the liver tissue (Fig. 8D and G). Such infiltrates were not seen in the WT mice.

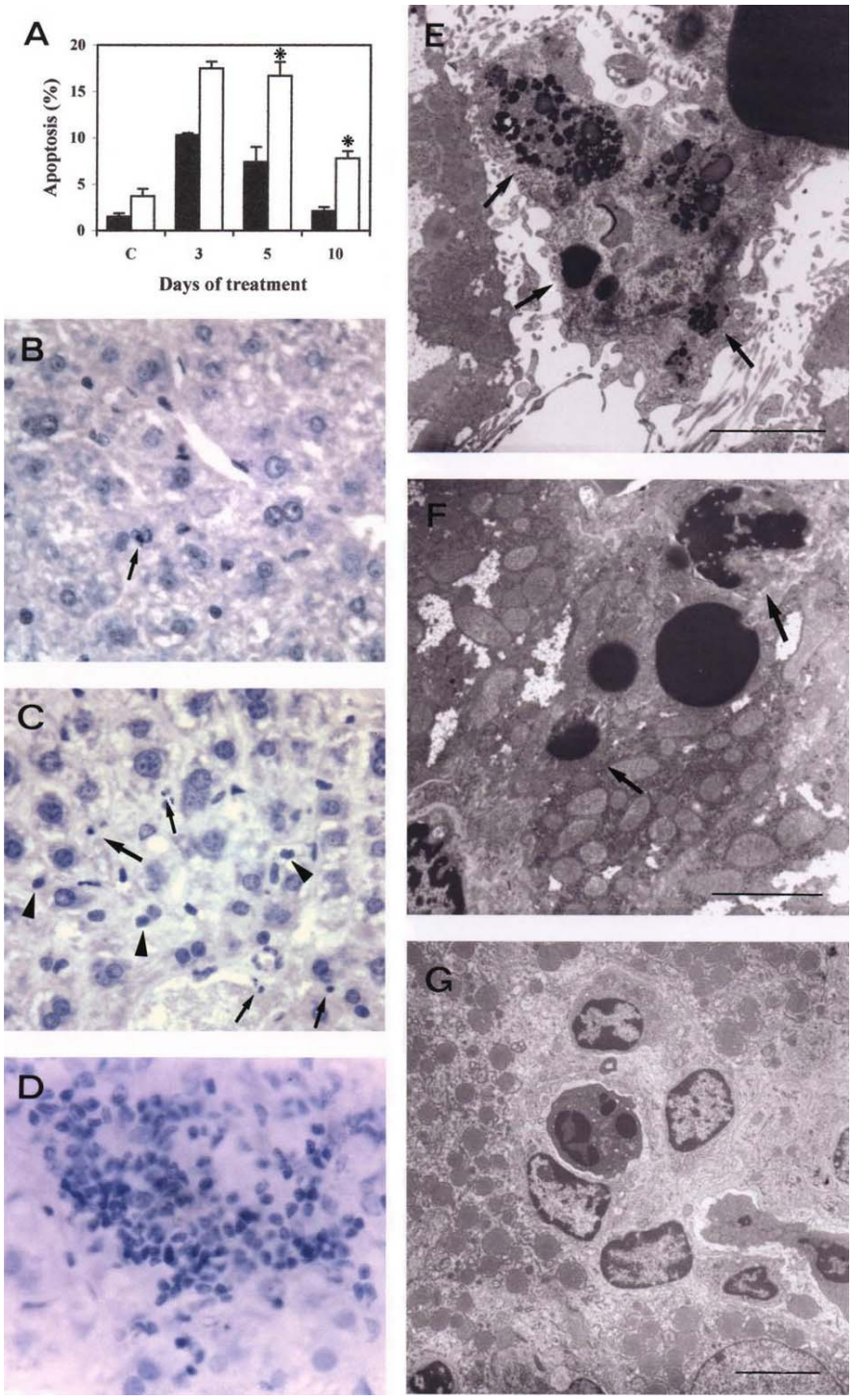

Figure 8 Morphological changes occurring in liver of wild-type and TGase2 null mice after $PbNO_3$ treatment. (A) Hepatocyte apoptosis in control and treated mice at 3, 5 and 10 days after injection of $PbNO_3$. Closed bars, wild-type; open bars, TGase2$^{-/-}$ livers. Values are presented as mean + SD of 3 independent experiments. *Statistically different from the wild-type mice ($p<0.05$). (B-D) Light microscopy of treated livers after 5 days. Haematoxylin-eosin-stained sections (original magnification 63X) demonstrate the presence of numerous apoptotic hepatocytes

(arrows) and massive inflammatory infiltrate in TGase2$^{-/-}$ (C-D), but not in wild-type liver (B). (E-G) Electron microscopic ultrastructural analysis of livers shows Kupffer cells actively engulfing apoptotic cells in wild-type mice as demonstrated by the presence of apoptotic remnants inside the cytoplasm (E, arrows). In contrast, in TGase2 null livers (F) the apoptotic bodies (arrows) not cleared by Kupffer cells fill the lumen of hepatic sinusoids and inflammatory cells, mostly lymphocytes and polymorphonuclear leukocytes, have infiltrated the liver parenchyma (G). Scale bar: 1μm (e), 2μm (F-G).

The production of active TGF-β1 promotes phagocytosis of apoptotic cells by macrophages and requires TGase2. To determine whether TGase2 in apoptotic cells or macrophages is required for proper phagocytosis, peritoneal macrophages were isolated from WT and TGase2$^{-/-}$ mice and exposed to apoptotic thymocytes in different combinations (Fig. 9A-D). Although, both WT and knock-out macrophages were competent in the phagocytosis of apoptotic thymocytes, the macrophages isolated from the knock-out mice showed a marked reduction in the average number of dead thymocytes found inside a cell (Fig. 9B, D and Table 2). The total number of apoptotic cells ingested within 1 h by 100 macrophages isolated from the knock-out mice was only 50 % of that of the WT animals. The deficiency seems to be selective for macrophages, as there was no difference in the phagocytotic efficiency when WT or knock-out thymocytes were used. Additionally, the deficit seems to be restricted to phagocytosis of apoptotic cells as TGase2$^{-/-}$ macrophages showed a normal ability to ingest bacteria, yeast or, as shown in **Table 2.**

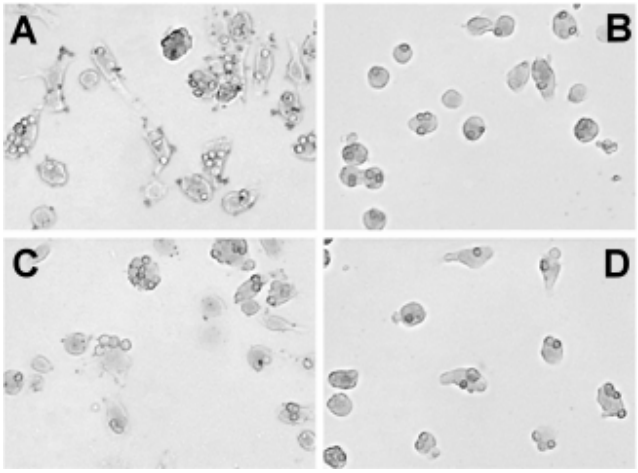

Figure 9 The rate of phagocytosis of apoptotic thymocytes by macrophages of TGase2$^{-/-}$ mice is only half of that of the wild-type mice. (A-B) Phagocytosis of wild-type thymocytes by wild-type and TGase2$^{-/-}$ macrophages, respectively. (C-D) Phagocytosis of TGase2$^{-/-}$ thymocytes by wild-type and TGase2$^{-/-}$ macrophages, respectively

.

Table 2 Phagocytosis of *L. monocytogenes,* Saccharomyces cerevisi and opsonized and non-opsonized thymocytes by wild-type and TGase2$^{-/-}$ macrophages

	Wild-type macrophages with ingested particles (%)	TGase2$^{-/-}$ macrophages with ingested particles (%)
Wild-type apoptotic thymocytes	76.7±8.2	51.1±4.7*
TGase2$^{-/-}$ apoptotic thymocytes	77.1±9.1	51.7±3.7*
Non-opsonized thymocytes	7.1±1.0	6.2±0.8
Thymocytes opsonized with anti-CD3 mAb	46.7±9.8	49.1±9.9
Thymocytes with isotype control mAb	14.1±2.3	11.7±1.6
L. monocytogenes	77.6±12.1	82.7±13.1
+50 μM cytochalasin B	17.6±5.2	14.7±6.9
Saccharomyces cerevisi	67.0±20.4	68.9±8.4
+50 μM cytochalasin B	22.8±11.7	24.7±14.1

Each experimental group included 3 independent experiments with macrophages isolated from separate mice. In the case of opsonized thymocytes cells treated with isotype control mAb served as control for nonspecific binding, while in the case of bacterial and yeast phagocytosis treatment with cytochalasin B, an inhibitor of phagocytosis, was used for discrimination of non-specific interaction with macrophages. Data represent mean ±S.D.

*Significantly different from the wild-type as determined by unpaired t-test ($p < 0.002$).

Macrophages are known to express TGase2 (Murtaugh MP *et al* 1984), which is required for the activation of latent TGF-β1 (Kojima S *et al.,* 1993), a cytokine specifically released by macrophages ingesting apoptotic cells. To test the possibility that the reduced rate of clearance of apoptotic cells observed in the TGase2$^{-/-}$ mice is related to a deficiency in TGF-β1 activation, apoptotic thymocytes were exposed to WT or TGase2$^{-/-}$ macrophages for one hour. The conditioned media were then collected and together with a fresh aliquot of apoptotic thymocytes added to TGase2$^{-/-}$ macrophages. Apoptotic cells were more efficiently ingested by TGase2$^{-/-}$ macrophages in the presence of conditioned medium derived from WT macrophages than in conditioned medium derived from TGase2$^{-/-}$ macrophages (**Table 3**) demonstrating that TGase2 participates in the production of a soluble factor that stimulates phagocytosis. Moreover, the inclusion of a neutralizing anti-TGF-β1,2,3 antibody was sufficient to inhibit this enhancement, demonstrating that the soluble factor was TGF-β.

Table 3 Deficit in the phagocytosis of apoptotic cells by TGase2$^{-/-}$ macrophages can be compensated by TGF-β activated by wild-type macrophages

	Phagocytosis of wild-type apoptotic thymocytes by	
	TGase2$^{-/-}$ macrophages in CM derived from wild-type macrophages	TGase2$^{-/-}$ macrophages in CM derived from TGase2$^{-/-}$ macrophages
	number of apoptotic bodies/100 macrophage	
	238±21*	126±17
+ a-TGF-β	129±18**	122±13
+ isotype specific mAb	247±22	128±15

The amount of phagocytosis was determined after 1h. Data represent mean±SD from four separate experiments.

CM, conditioned medium collected after 1h of phagocytosis of apoptotic cells by wild-type or TGase2$^{-/-}$ macrophages as indicated; a-TGF-β, anti-TGF-β 1,2,3 neutralizing monoclonal antibody (R&D Systems, 10 μg/ml); isotype specific mAb, mouse IgG1, control for the neutralizing monoclonal antibody (Pharmingen, 10 μg/ml).

*Significantly different from the one observed in CM derived from TGase2$^{-/-}$ macrophages determined by unpaired t-test ($p<0.002$).

**Significantly different from the one observed in the absence of the neutralising mAb determined by unpaired t-test ($p<0.002$).

TGF-β1 is responsible for the induction of TGase2 expression during in vivo apoptosis. TGF-β1 can induce TGase2 expression via a TGF-β response element in its promoter (Ritter SJ and Davies PJ 1998). As our previous experiments suggested that the *in vivo* up-regulation of TGase2 requires unknown factors present only in the tissue environment (Szegezdi E *et al.,* 2000), and the TGase2 promoter in the TGase2$^{-/-}$ thymus, in contrast to wild-type thymus, was only marginally induced during *in vivo* apoptosis (Fig. 7A), we asked the question whether active TGF-β could be one of the unknown tissue mediators. If this is the case, injection of neutralizing anti-TGF-β1,2,3 antibodies should reduce the up-regulation of TGase2 in wild-type mice. Indeed, while the induction of the TGase activity as compared to the physiological saline treated littermates was 1.7±0.2, 3.8±0.3 and 4.7±0.4-fold following anti-CD3, DXM treatment or γ–irradiation, respectively, in the presence of anti-TGF-β antibodies the induction was only 1.3±0.1, 2.0±0.2 and 3.1±0.2 fold. In each treatment group, the increase in TGase activity was significantly reduced in those mice which received the neutralizing anti-TGF-β antibodies ($p<0.05$, $n=4$). The same dose of isotype matched unrelated antibody had no effect (Fig. 10).

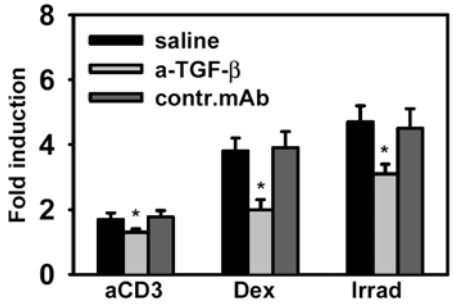

Figure 10 Injection of anti-TGF-β monoclonal antibody impairs the induction of TGase activity in the thymus following apoptosis induction with various stimuli. Data represent mean±SD of four mice. *Significantly different from the TGase activity of mice, which did not receive the anti-TGF-β antibody determined by unpaired t-test ($p<0.05$)

TGF-β1 promotes apoptosis of thymocytes. Since the *ex vivo* rate of thymocyte apoptosis of TGase2$^{-/-}$ mice was not altered (De Laurenzi V and Melino G 2001; Nanda N. *et al.,*2001), but the *in vivo* rate was slower (Fig. 7), and induction of the expression of TGase2 has been shown to promote apoptosis (Melino G 1994), we tested whether TGF-β1 would promote the rate of cell death in *ex vivo* thymocyte cultures. Addition of TGF-β1 which by itself induced only a 2.1±0.3 % increase in the 5.1±1.1 % rate of spontaneous death of WT thymocytes, promoted cell death induced by 0.1 μM DXM, 10 μg/ml anti-CD3 mAb and 5 μM etoposide by 26.4±2.9, 13.1±1.6 and 26.7±2.2 %, respectively (each of them significantly different from the control value $p<0.05$, $n=4$) as detected after 6 h of culture (Fig. 11). This effect of TGF-β1, however, was not related to the up-regulation of TGase2 in apoptotic cells, because it promoted thymocyte apoptosis in TGase2$^{-/-}$ mice to a similar degree.

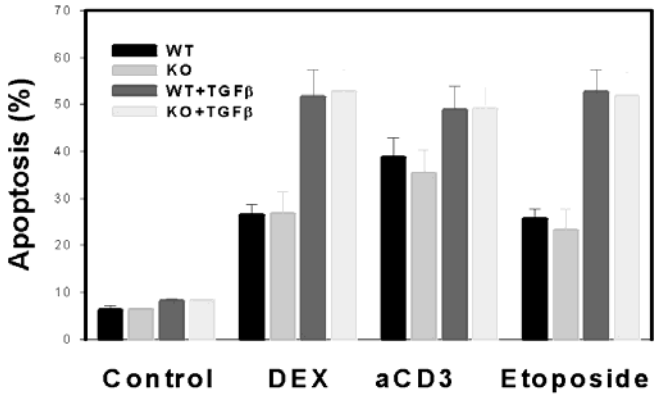

Figure 11 TGF-β1 promotes the rate of cell death induced by DXM, anti-CD3 and etoposide in *ex vivo* thymocyte cultures independently of TGase2 up-regulation.

TGase2$^{-/-}$ mice develop autoantibodies, splenomegaly and immune complex glomerulonephritis. We then investigated the long-term effects of the TGase2 deficiency. We found that 9 out of 25 one year old TGase2$^{-/-}$ mice had autoantibodies against nuclear components, and 2 (one with and one without antinuclear positivity) produced autoantibodies against smooth muscle cells (Fig. 12A), while none of the 20 age matched control wild-type mice tested positive. Moreover, the 10 mice with autoantibodies developed splenomegaly with an average weight of 167±98.2 mg as compared to the 92.3±19.1 mg in controls. While there was no change in the total number of $CD8^+$ T cells in the enlarged spleens, the total number of $CD4^+$ cells was significantly decreased ($13\pm3x10^6$ versus the normal $25\pm4x10^6$) and that of the B cells significantly increased ($240\pm60x10^6$ versus the normal $160\pm35x10^6$ $p<0.05$, n=10). These spleens showed an enlarged white pulp characteristic for hyperactive spleens (Fig. 12B). Besides the anti-nuclear and anti-smooth muscle antibodies, increased titers of IgG type anti-IgG2a antibodies were detected in 18 out of the 25 tested knock-out animals. For these 18 animals, a mean OD492 of 0.310±0.112 was measured by ELISA as compared to 0.071±0.022 (n=15) in the wild-type animals, ($p<0.002$). However, 5 out of the 20 wild-type mice had also elevated titers. Elevated titers of anti-IgG1 antibodies were also detected in 5 out of the 9 antinuclear positive knock-out mice and in one of the anti-nuclear antibody negative mice (data not shown), while no anti-IgG1 antibodies were found in the wild-type mice. At the age of 15 months, 7 out of 10 knock-out mice demonstrated antinuclear antibodies and one anti-cytoskeletal positivity, and 5 out of these were terminally ill with kidney disease diagnosed as immune complex glomerulonephritis (Fig. 12C). Glomerular dysfunction was indicated by high serum concentrations of urea (31 to >100 mM versus the normal 11±3 mM). At the same age, 3 out of 15 wild-type animals tested showed antinuclear positivity but none had signs of kidney damage.

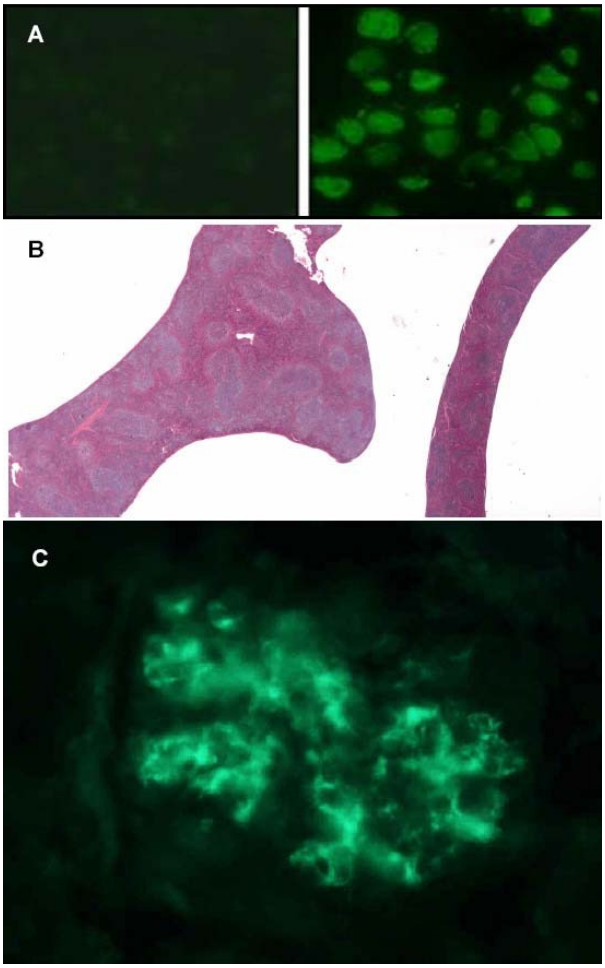

Figure 12 Prevalence of autoantibodies, splenomegaly and glomerulonephritis in TGase2$^{-/-}$ mice. (A) Detection of antinuclear antibodies in the sera of TGase2$^{-/-}$ mice (40x diluted) using rat liver sections. The left panel demonstrates a weak reaction to nuclear components, while the right one shows a strong anti-nuclear reactivity. (B) Light-microscopic images of sections of one year old and TGase2$^{-/-}$ (left) and wild–type (right) mice spleens stained with haematoxylin-eosin. The weights of the spleens were 413 and 96 mg, respectively. Original magnification 6 X. (C) The presence of IgM-containing immune complexes in glomeruli, revealed by staining frozen kidney sections with FITC-labelled goat anti-mouse IgM antibodies.

Loss of TGase2 does not affect the rate of thymocyte apoptosis in vitro from exposure to anti-Fas antibodies, though the rate observed in vivo is slightly delayed. Previous work in our laboratory has shown that intraperitoneal administration of anti-Fas antibodies at a dose of 1μg/g body weight does not kill wild-type mice, but the rate of Fas-mediated thymocyte apoptosis *in vivo* can be studied (Szondy Z *et al*., 1997a). To monitor the rate of thymocyte apoptosis *in vivo*, the decrease in the percentage of $CD4^+CD8^+$ (apoptosis-sensitive) population were determined in

the wild-type and in knock-out animals after 20 hrs following anti-Fas injection. As shown in Figure 13, following anti-Fas injection, the size of the $CD4^+CD8^+$ thymocyte pool decreased in both type of the animals, and the disappearance of $TGase2^{-/-}$ thymocytes was slightly delayed. However, no significant difference was found in the rate of apoptosis *in vitro* induced by anti-Fas antibodies determined at 24h, which was 56.5±4.7 and 55.4±3.9 % in the wild-type and knock-out thymocytes, respectively. These data suggest that loss of TGase2 does not affect Fas sensitivity of $TGase2^{-/-}$ thymocytes. During the experiments we noticed that the $TGase2^{-/-}$ mice are more susceptible to the anti-Fas antibody treatment i.e. they did not survived the otherwise for the wild-type mice sub-lethal dose of anti-Fas. We decided to characterize this phenomenon.

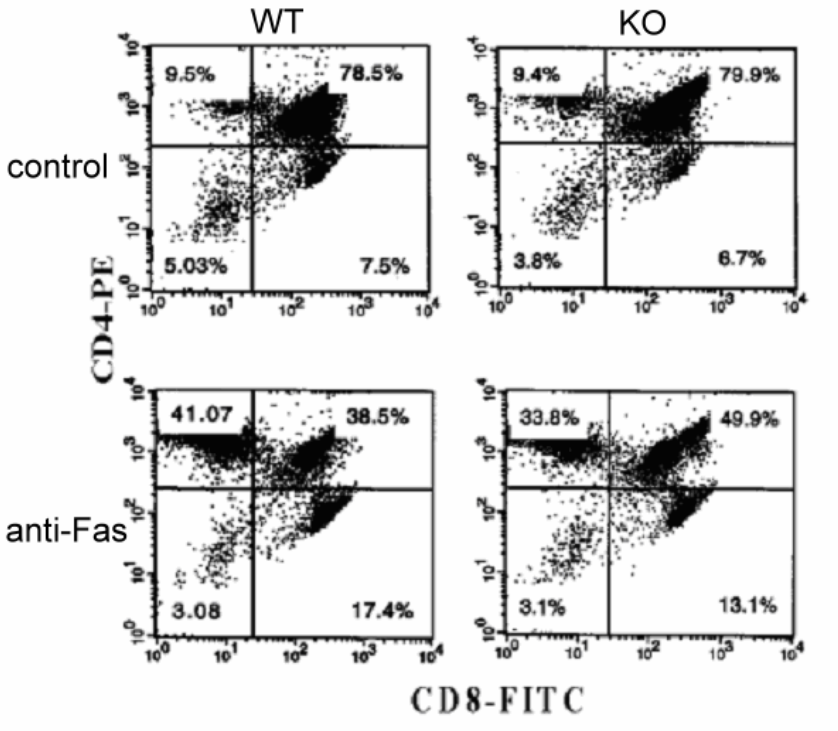

Figure 13 Effect of a single dose of intraperitoneal injection of anti-Fas antibodies on the apoptosis of thymocytes of wild-type and $TGase2^{-/-}$ mice. Mice were treated intraperitoneally with 1 μg/g body weight of anti-Fas antibodies and the changes in the percentage of various thymic subpopulations were determined by FACS analysis at 20h following treatment.

$TGase2^{-/-}$ animals are more sensitive to Fas-induced killing than wild-type animals

Despite of the lack of changes in Fas sensitivity of thymocytes, or even the delayed rate of their death *in vivo*, $TGase2^{-/-}$ mice were much more susceptible to Fas-induced killing. While all the

wild-type animals survived the 1μg/g body weight intraperitoneal dose of Jo2 antibody, none of the knock-outs survived for more than 20 hrs following administration of the same dose of anti-Fas antibodies (Fig. 14).

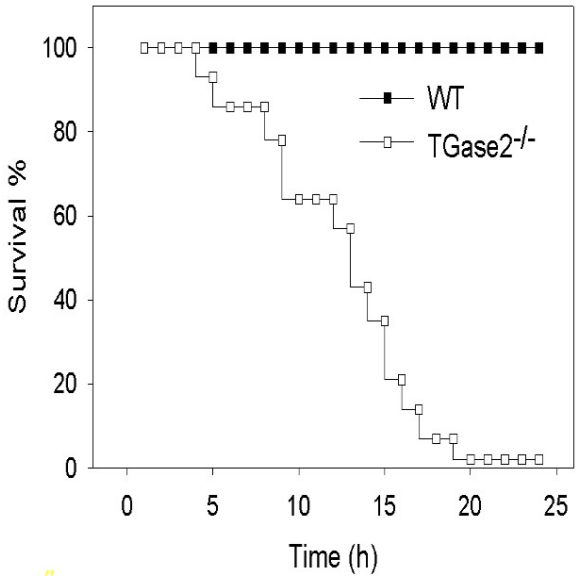

Figure 14 Effect of a single dose of intraperitoneal injection of 1 μg/g body weight anti-Fas antibodies on the survival of wild-type and TGase2$^{-/-}$ mice.

Wild-type hepatocytes die primarily by necrosis, while TGase2 knock-out cells die by apoptosis following Fas engagement in vivo by a non-lethal dose of anti-Fas antibodies. Since it was previously shown that higher doses of anti-Fas antibodies kill mice via inducing massive liver apoptosis (Ogasawara J *et al*., 1993), histological sections of livers from both wild-type and TGase2$^{-/-}$ mice were investigated following anti-Fas antibody injection. In the wild-type liver at 5h following anti-Fas injection, the structure investigated by light microscope (LM) seemed to be only slightly altered, as compared to the un-treated controls (Fig. 15A), with dilated central veins visible and with perivenous hepatocytes displaying a pale, granulated cytoplasm (Fig. 15C). Electron microscopically, however, necrotic hepatocytes could be detected displaying vacuolized plasma and disorganized mitochondria (Fig. 15C). 19 hrs later significant cellular damage could be detected by LM, with markedly dilated blood vessels, singular-cell or confluent necrosis with a marked hemorrhagic component, and with a few apoptotic cells being visible (Fig. 15D). These changes were significantly more conspicuous when the samples were examined by transmission electron microscopy (TEM) (Fig. 16E).

Within the sections of livers from the non-treated knock-out animals no alterations in the liver structure were found even at electron microscopical level (Fig. 16B). However, at 5 hrs following Fas-engagement *in vivo*, when the animal died, a significantly increased number of apoptotic hepatocytes could be detected by LM (Fig. 15B). This was accompanied by massive hemorrhagic suffusions, suggesting also the development of disseminated endothelial cell damage. Appearance of apoptotic cells was confirmed by TEM (Fig. 16D). In addition, a peculiar finding is demonstrated in Figure 16F. In several samples, the formation of labyrinthine, curvilinear structures that resemble folded up or spiraling rough/smooth endoplasmic reticulum was detected. These structures often appeared in isolation from hepatocytes, without a clearly definable outer limiting membrane.

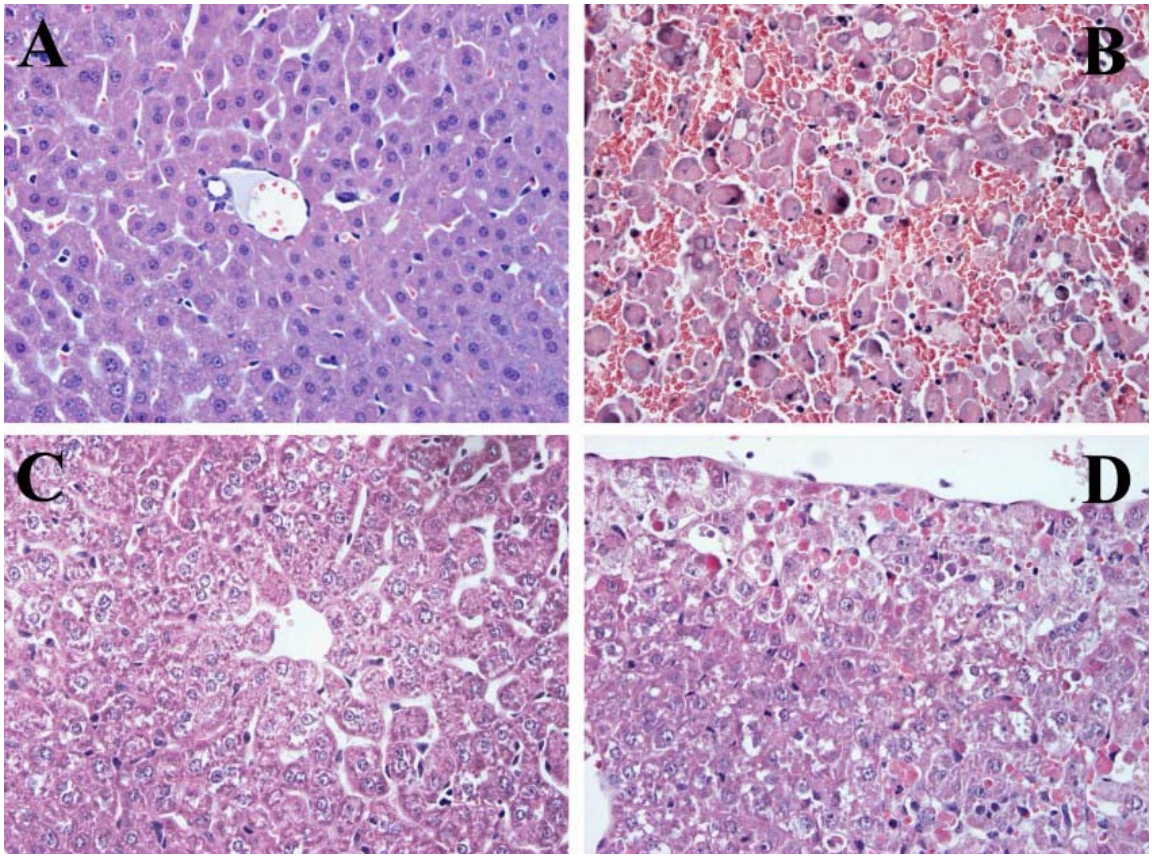

Figure 15 Representative photomicrographs of livers obtained from 4 weeks old control (A), anti-Fas-treated wild-type (C 5h, D 24h) or anti-Fas-treated TGase2$^{-/-}$ (B 5h) mice. Hematoxylin-eosin stains show mild (C) and severe (D), predominantly necrotic cellular changes with accumulation of Mallory bodies in the livers of wild-type mice following 5 and 24 hrs of anti-Fas-treatment, respectively. In the liver of TGase2$^{-/-}$ mice (B), however, hepatocytes die by apoptosis, and this is accompanied by massive hemorrhagic suffusions due to endothelial cell lesions (original magnification 400x).

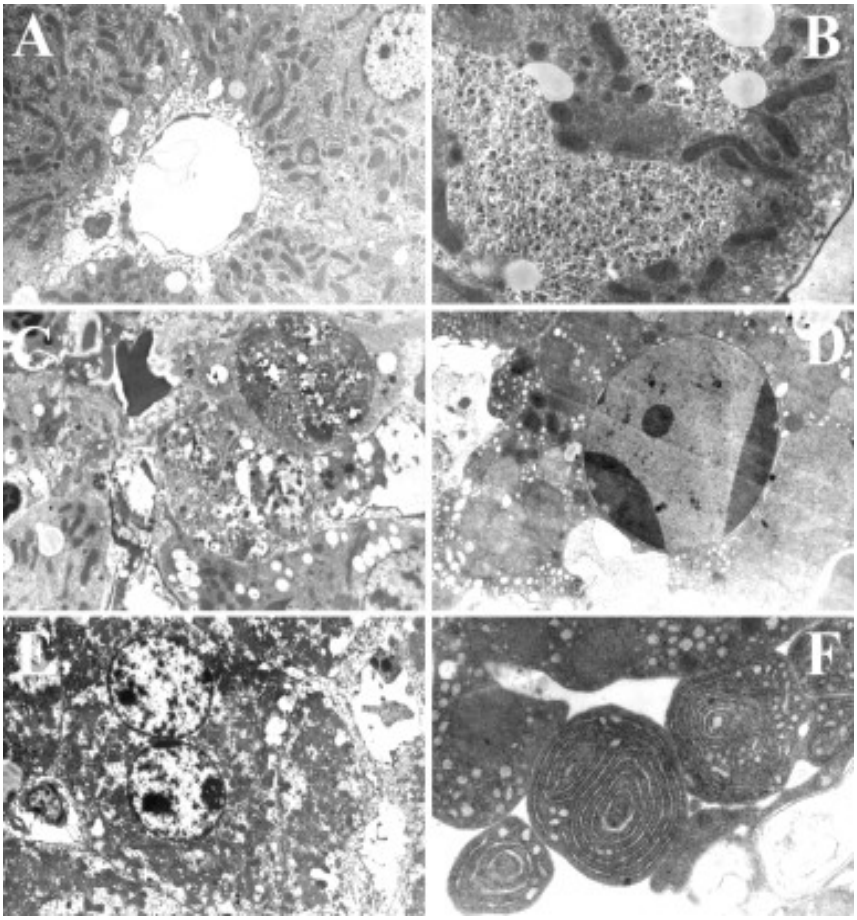

Figure 16 Electron microscopic ultrastructural analysis of livers of non-treated wild-type (A) or TGase2$^{-/-}$ (B), and anti-Fas-treated wild-type (C 24h, E 5h) or TGase2$^{-/-}$ (D,F 5h) mice. In wild-type livers 5 hrs of Fas treatment (E) results in mild granular-vacuolar degeneration of cells, while at 24h (C) severely damaged hepatocytes can be detected with obscured chromatin, vacuolated cytoplasm and phagolysosomes. The distorted red blood cell within the sinusoid demonstrates endothelial cell damage. While the ultrastructure of the liver of non-treated knock-out animals is unaltered (B), apoptotic cells (D) sometimes with unusual morphology (F) appear following anti-Fas-treatment. Original magnification 3,000x (A,C,E), 8,000x (B), 6,000x (D) or 12,000x (F).

In support of this, whilst in the livers of knock-out animals massive hemorrhagic suffusions were detected by HM staining (Fig. 15B), accompanied with numerous apoptotic nuclei, detected by TUNEL staining, all over the tissue (Fig. 17D) already at 5h following anti-Fas treatment, at the same time point only the endothelial cells were stained positive for apoptotic nuclei in the wild-type livers (Fig. 17A and B). In addition, in accordance with the observation that wild-type hepatocytes died primarily by necrosis, very few apoptotic hepatocytes were detected, even at 24h following anti-Fas antibody injection (Fig. 17C).

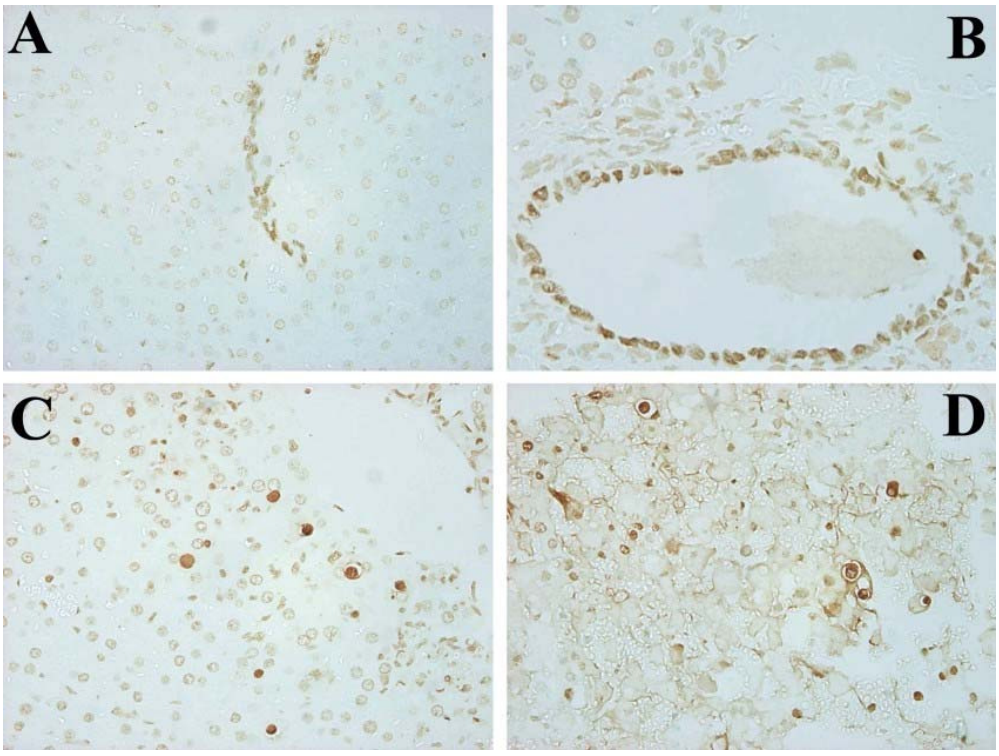

Figure 17 Immunohistochemical detection of apoptotic nuclei in the liver sections of anti-Fas-treated wild-type and TGase2$^{-/-}$ mice. In the wild-type livers removed at 5h following anti-Fas treatment mostly endothelial cells are stained (A,B), and even at 24h only very few hepatocytes are positive (C). In contrast, TGase2$^{-/-}$ livers (D) are full with apoptotic nuclei already at 5h (original magnification 400x).

The increased damage of TGase2$^{-/-}$ hepatocytes following anti-Fas treatment *in vivo* was further confirmed by the significantly elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in KO mice, determined at points of the death, as compared to wild-types at the time points of the death of knock-out animals.

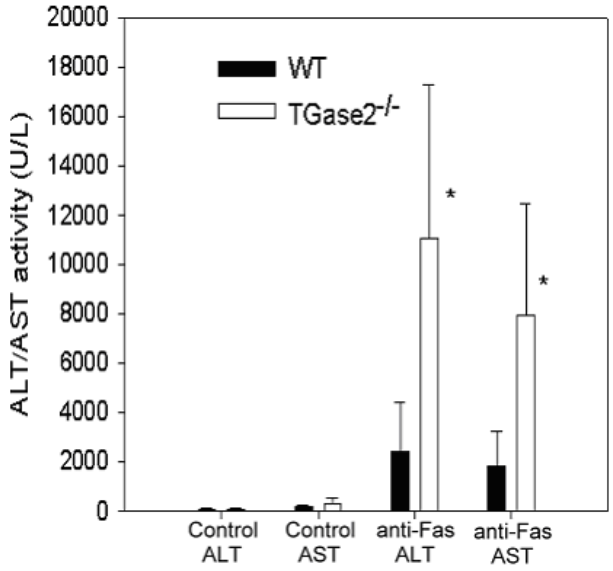

Figure 18 Wild-type and TGase2$^{-/-}$ mice were treated intraperitoneally either with saline or anti-Fas antibodies (1 μg/g body weight). Serum ALT and AST levels were determined in the case of knock-out animals at the time point of their death, while in the case wild-types at the same time points when the knock-outs died. Values are expressed as mean ± SD (n=5 for the saline treated control, while n=15 for the two other anti-Fas-treated groups). *Statistically different from the anti-Fas treated WT mice as determined by nonpaired t-test ($p<0.05$).

These data implied that TGase2$^{-/-}$ livers are more sensitive to an otherwise non-lethal dose of anti-Fas antibodies, that the type of hepatocyte death induced by the non-lethal dose of anti-Fas antibodies is different in the wild-type and knock-out animals, and TGase2, as a cross-linking enzyme, might be required for the proper apoptotic morphology.

TGase2$^{-/-}$ hepatocytes show increased sensitivity towards anti-Fas treatment in vitro in correlation with their lower Bcl-x_L expression. To determine whether TGase2$^{-/-}$ hepatocytes are more sensitive to anti-Fas treatment than wild-type cells also *in vitro*, hepatocytes were isolated, cultured and exposed to increasing concentrations of anti-Fas antibodies. The percentage of apoptotic hepatocytes was determined 24 hrs later. As shown in Figure 19A, wild-type hepatocytes survived well under the experimental conditions and were relatively resistant to Fas-mediated death, as shown previously (Ni R *et al*., 1994). TGase2$^{-/-}$ hepatocytes, on the other hand, demonstrated both an increased rate of spontaneous death in culture and an increased sensitivity to Fas-mediated death, as compared to wild-type cells. The increased sensitivity was not related

to an enhanced cell surface expression of Fas, since no difference in the cell surface expression of the receptor was detected between wild-type and TGase2$^{-/-}$ hepatocytes (Fig 19B).

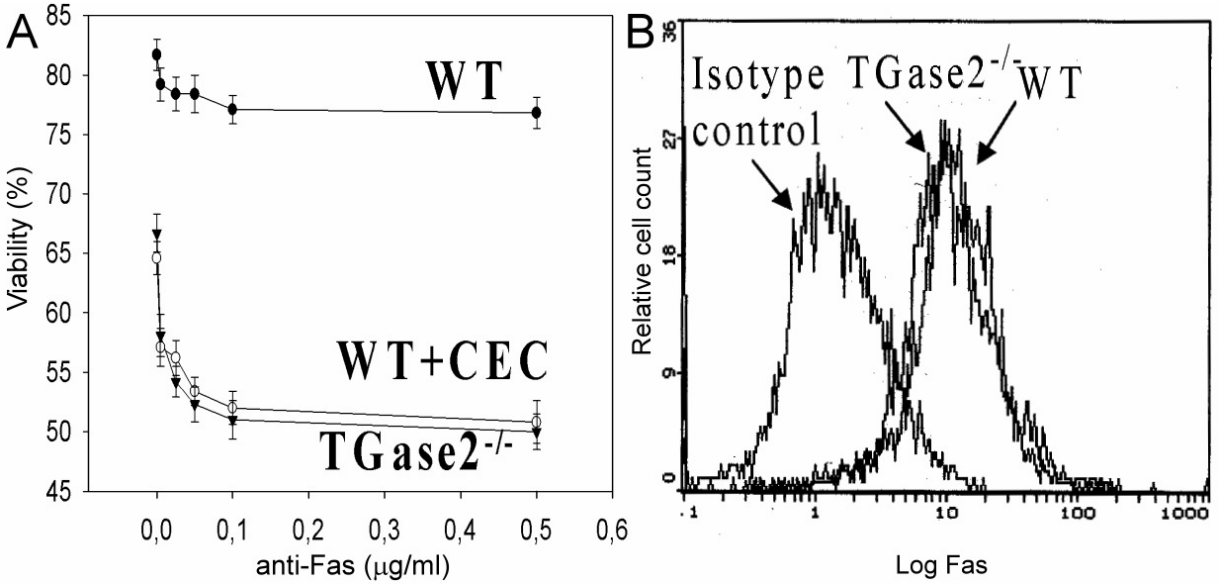

Figure 19 *In vitro* Fas sensitivity (A) and Fas cell surface (B) of wild-type and TGase2$^{-/-}$ hepatocytes. Primary mouse hepatocytes were isolated from wild-type, chloroethylclonidine-treated (daily dose of 25, 50, 75, 100 μg for a week) wild-type and TGase2$^{-/-}$ mice. Fas expression was determined by FACS. For the viability experiments wild-type mice were pretreated with 100 μg daily dose of chloroethylclonidine (CEC). Data represent mean ± SD (n=3).

Because TGase2$^{-/-}$ hepatocytes died spontaneously at a higher rate, and the spontaneous death-rate is known to be influenced by the levels of anti-apoptotic proteins, the levels of Bcl-x_L and cFLIP(L) expression in hepatocytes were also determined. As shown in Figure 20A, Bcl-x_L expression was found to be decreased in TGase2$^{-/-}$ hepatocytes, as compared to the wild-type cells. There was no difference in cFLIP(L) expression between the wild-type and KO mice (Fig 24B).

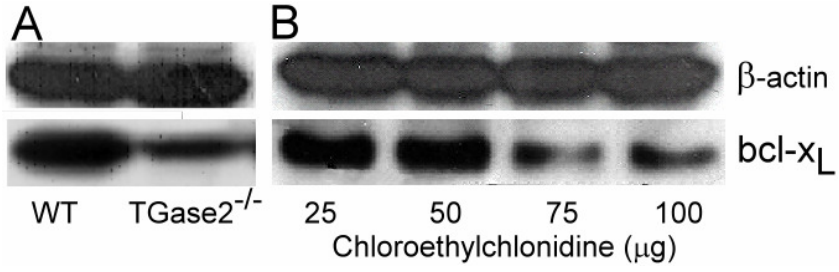

Figure 20 Western blot analysis of Bcl-x_L expression of untreated (A) wild-type and TGase2$^{-/-}$ hepatocytes. Primary mouse hepatocytes were isolated from wild-type, chloroethylclonidine-treated (daily dose of 25, 50, 75, 100 μg for a week) wild-type and TGase2$^{-/-}$ mice.

Inhibition of the alpha-1-β-adrenerg receptor leads to both down-regulation of the Bcl-x_L expression and increased Fas-sensitivity of hepatocytes. Since TGase2 participates as a G protein in the ADR-signalling pathway in the liver, we decided to test the possibility that impaired ADR signal transduction is related to the observed decrease in Bcl-x_L expression in the liver of TGase2$^{-/-}$ mice. To achieve this, various doses of chloroethylclonidine, a α1-b adrenoceptor antagonist, was injected daily in wild-type mice for a week, and than the Bcl-x_L expression of hepatocytes was determined. As shown in Figure 20B, inhibition of the ADR signalling pathway by chloroethylclonidine resulted in a dose-dependent decrease in the Bcl-x_L expression of hepatocytes. When hepatocytes pretreated *in vivo* with chloroethylclonidine were isolated and cultured, their sensitivity to anti-Fas treatment *in vitro* and the rate of their spontaneous cell death were similar to that of TGase2$^{-/-}$ hepatocytes (Fig. 19A). These data demonstrated the impaired ADR signalling, which develops also in the absence of TGase2, can lead to down-regulation of Bcl-x_L and to a consequent increase in Fas sensitivity.

α_1-b adrenergic receptor knock-out mice are more susceptible to Fas-induced killing than their wild-type counterparts. To prove further the role of ADR signalling in the regulation of Fas sensitivity of hepatocytes, the Fas sensitivity of α_1-b adrenoceptor deficient mice (Cavalli A *et al*., 1997) was also determined. The wild-type partners for the ADR deficient mice were C57BL/6 mice, in which the hepatic density of β_2-adrenergic receptors is low. As a result these mice were reported to exhibit increased sensitivity to Fas mediated apoptosis of hepatocytes (Andre C *et al*., 1999) Consequently lower doses of anti-Fas antibodies were found to be sublethal for this mouse strain. Injection of 0.3 μg/g body weight antibody resulted in 80% survival of the wild-type (C56BL/6) mice (Fig. 21). The same dose of antibody, however, killed 90% of the AR deficient mice within two days.

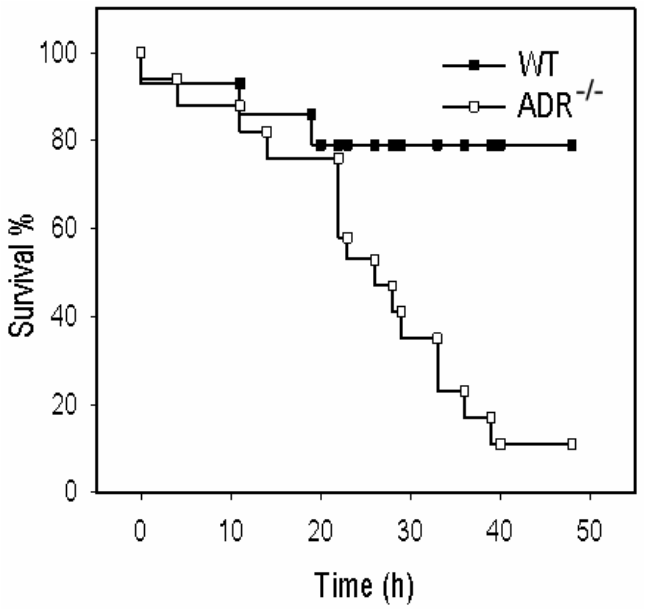

Figure 21 Effect of a single dose of intraperitoneal injection of 0.3 μg/g body weight anti-Fas antibodies on the survival of wild-type and α1-b adrenergic receptor knock-out mice.

Livers from α_1-b adrenergic receptor deficient mice show considerably more damage than wild-type livers after anti-Fas engagement. Since there was a significant difference between the death rate of WT and α_1-b adrenergic receptor knock-out mouse strains after 0.3 μg/g body weight anti-Fas treatment we continued with the histological examination of the livers. Livers were examined in case of the $ADR^{-/-}$ mouse after the time of death (28h) or in case of WT mouse at the time point when the KO mouse died. LM sections of Fas-treated $ADR^{+/+}$ livers revealed that besides the healthy looking parenchymal cells many hepatocytes lost their nucleus, contained nuclear fragments and their cytoplasm was condensed, homogenous, eosinophilic (Fig. 22A). These cells represented apoptotic cells. A low number of necrotic cells were also visible. On the LM sections of Fas-treated $ADR^{-/-}$ livers the basic structural features of the histological structure of liver tissue were mostly lost. The central veins were discernible, but the sinusoids were not. The majority of parenchymal cells either lost their nucleus, or showed apoptotic morphology. In addition, necrotic cells were also visible (Fig. 22B). Increased damage of adrenoceptor deficient livers as compared to their wild-types was demonstrated by the higher number of TUNEL positive liver cells on tissue sections (Fig. 22C and D).

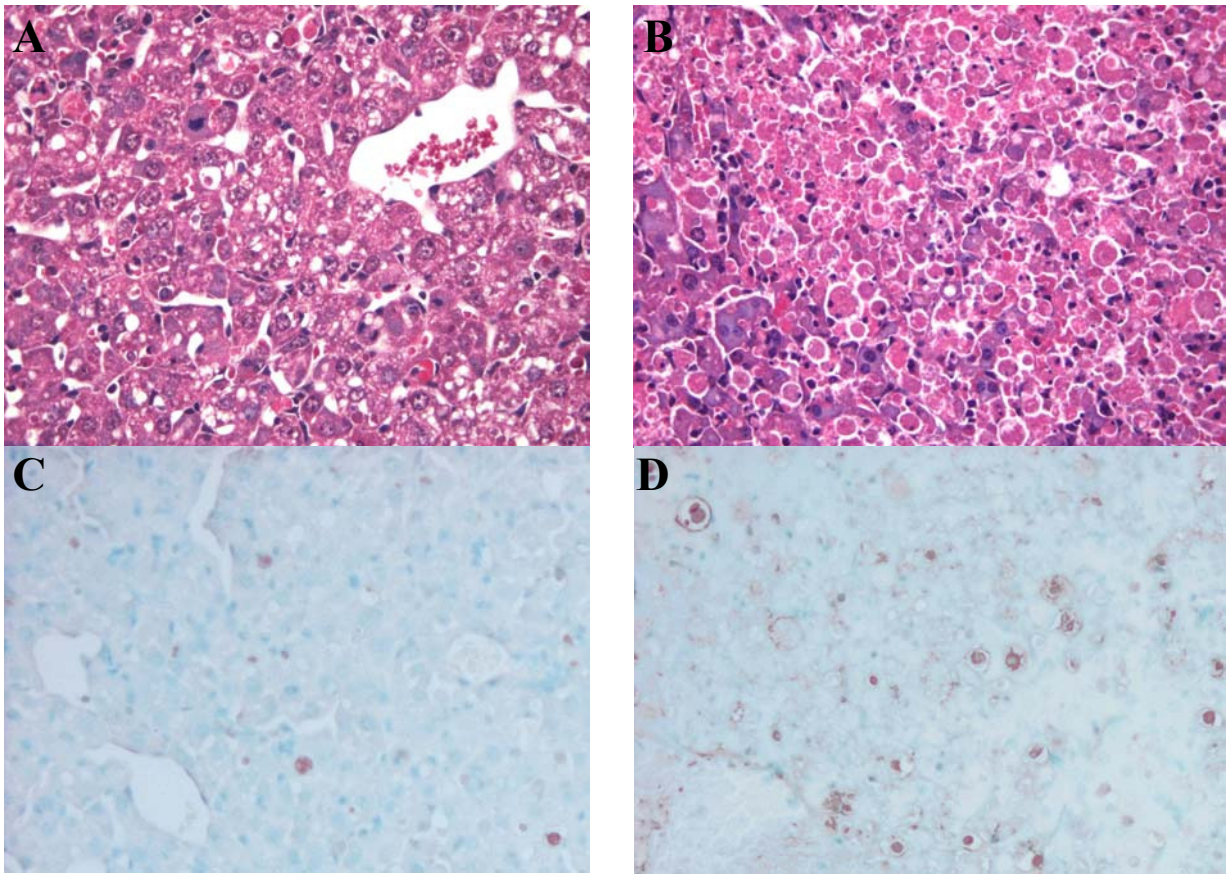

Figure 22 Representative photomicrographs of livers obtained from 4 weeks old wild-type (A, C) or α1-b adrenergic receptor knock-out (B, D) mice 28h after Fas agonist injection. Hematoxylin-eosin and TUNEL stains show mild (A, C) and severe (B, D) parenchymal degradation with accumulation of apoptotic bodies in the livers of wild-type and knock-out mice following 28 hrs of anti-Fas-treatment, respectively (original magnification 400x).

The increased damage of α_1-b adrenergic receptor knock-out hepatocytes following anti-Fas treatment *in vivo* was further confirmed by the significantly elevated serum AST and ALT levels in KO mice (Fig. 23), determined at points of the death, as compared to wild-types at the time points of the death of knock-out animals.

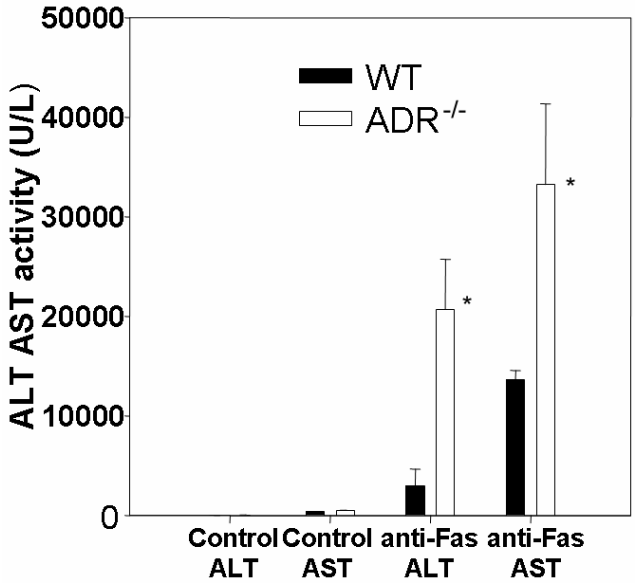

Figure 23 Wild-type and α_1-b adrenergic receptor knock-out mice were treated intraperitoneally either with saline or anti-Fas antibodies (0.3 μg/g body weight). Serum ALT and AST levels were determined in the case of knock-out animals at the time point of their death, while in the case wild-types at the same time points when the knock-outs died. Values are expressed as mean ± SD (n=3 for the saline treated controls and the anti-Fas-treated WT mice, while n=5 for the anti-Fas-treated KO mice). *Statistically different from the anti-Fas treated WT mice as determined by nonpaired t-test ($p<0.05$).

Next, we investigated whether the increased Fas sensitivity of the $ADR^{-/-}$ strain was also a consequence of a reduced Bcl-x_L expression in hepatocytes. α_1-b adrenergic receptor deficient hepatocytes, similar to $TGase2^{-/-}$ hepatocytes, expressed less Bcl-x_L than their WT counterparts did (Fig. 24A). There was no difference between the cFLIP(L) expression of ADR WT and KO hepatocytes although both strains expressed lower amount of cFLIP(L) than the TGase2 WT and KO mice (Fig. 24B).

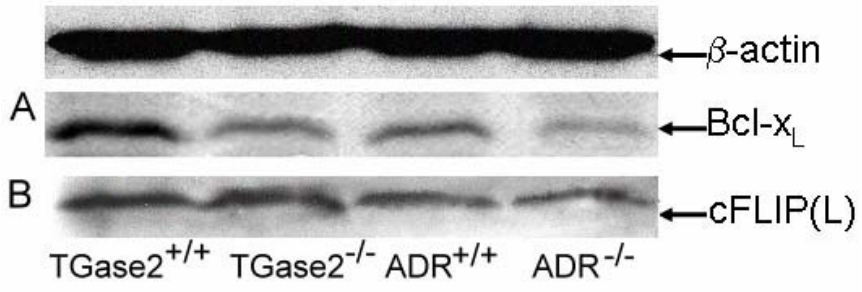

Figure 24 Western blot analysis of Bcl-x_L and cFLIP(L) expression of $TGase2^{+/+}$, $TGase2^{-/-}$, adrenergic receptor WT and α_1-b adrenergic receptor deficient hepatocytes.

Discussion

TGase2 upregulation is associated with the *in vivo* apoptosis program in many cells (Fesus L *et al*., 1987c). In my thesis, I investigated the impact of the absence TGase on apoptosis. In order to clarify the role of TGase2 in apoptosis mice lacking TGase2 have been generated by homologous recombination techniques. These mice reproduced normal, showed no major developmental abnormalities, and histological examination of the major organs appeared normal. Induction of apoptosis *ex vivo* in TGase2$^{-/-}$ thymocytes (by CD95, dexamethasone, etoposide, and H_2O_2) and *in vitro* on TGase2$^{-/-}$ mouse embryonal fibroblasts (by retinoids, UV, and H_2O_2) showed no significant differences and only a minor reduction in the formation of cross-linked apoptotic bodies was detected during UV or H_2O_2 induced apoptosis (De Laurenzi V, Melino G, 2001). These authors concluded that loss of TGase2 does not influence the *in vivo* apoptosis program.

In this study, we investigated both the *in vitro* and *in vivo* apoptosis program. Though the presence of the enzyme promoted or, in some cells, induced apoptosis, the apoptotic program can be induced in the absence of TGase2 activity, and *in vitro* many cells die without the apparent upregulation of the enzyme. Our finding, in agreement with previous results (De Laurenzi V, Melino G, 2001), was that loss of TGase2 did not affect the *in vitro* apoptosis of thymocytes. This can be explained by the fact that the same apoptotic stimuli that upregulate the enzyme in the thymus *in vivo,* fail to do so *in vitro* (Szegezdi E *et al*., 2000). However, after *in vivo* apoptosis induction, where TGase2 is normally upregulated, a reduced cell death was detected in the thymi of TGase2 knock-out mice (Fig. 7D, E and F). This might be the result of the fact that in the absence of TGase2 thymocytes activate they apoptosis program slower. TGase2 was shown to sensitize cells towards apoptosis by the hyperpolarization of mitochondrial membrane (Piacentini, M. *et al.,* 2002) in addition TGase2 can induce oligomerization of Bax that results in the stabilization of its pore forming conformation, accelerating the release of cytochrome-c, during apoptosis (Rodolfo C *et al*., 2004). Secondly, we have found that addition of recombinant TGF-β to thymocyte cultures promotes their death. Since TGF-β can be released by macrophages ingesting apoptotic cells, TGF-β production *in vivo* might contribute to the *in vivo* apoptosis rate. In the absences of TGase2 TGF-β activation is impaired (Kojima S *et al*., 1993) and this might lead to slower apoptosis rate.

Alteration of the rate of cell death was not restricted to the thymus. Treatment of WT and TGase2 knock-out mice with anti-Fas, as a T-cell apoptosis inducing agent, revealed another consequence of TGase2 deficiency. The liver of mice is particularly sensitive *in vivo* to the anti-Fas antibody Jo2, which cause massive liver apoptosis that is followed by the death of the animals after only a few hours, with a phenotype that resembles fulminant hepatitis (Ogasawara J *et al*., 1993). Here we show that TGase2 deficient mice display increased sensitivity to Fas mediated death as all TGase2 knock-out mice died within 24 hrs after anti-Fas treatment while their WT counterparts survived. Elevated serum AST and ALT levels in TGase2$^{-/-}$ mice confirmed liver damage. Histological examination of the livers revealed that in the absence of TGase2 hepatocytes died rapidly after anti-Fas engagement primary by apoptosis. Hemorrhagic suffusions indicated endothelial cell damage as well. In contrast, in the liver of WT animals, predominantly endothelial cell apoptosis could be observed at early time point after anti-Fas antibody treatment and hepatocytes showed necrotic morphology probably because of the oxygen and nutrition depletion due to the endothelial cell damage and microcirculation failure after 24 hrs. The increased Fas sensitivity could be also detected on isolated hepatocytes implying intrinsic changes in the Fas-mediated cell death pathway. Therefore, we investigated the cell surface Fas expression and found that the increased Fas sensitivity was not related to increased amount of Fas on TGase2 hepatocyte. cFLIP(L), which in high concentration can block the Fas-triggered apoptotic pathway (Irmler M *et al*., 1997), was also expressed at the same level in WT and TGase2$^{-/-}$ hepatocytes. Hepatocytes belong to type II cell in which apoptosis occurs involving the mitochondrial pathway. Since Bcl-x_L is present in adult rat liver and in cultured mouse hepatocytes (Rouayrenc JF *et al*., 1995) and its overexpression was reported to be protective against Fas induced cell death (de la Coste A *et al*., 1999) we have investigated Bcl-x_L expression and found that TGase2$^{-/-}$ hepatocytes expressed less Bcl-x_L than WT cells.

TGase2 as $G_{alpha(h)}$ protein was shown to mediate ADR signalling in the liver (Wu J. *et al*., 2000). In hepatocytes from adult animals the predominant adrenergic receptor is the α_1-b-subtype, although the β2-subtype receptors also are present on the same hepatocytes. Adrenergic agonists modulate not only the metabolism but can act as mitogens in hepatocytes; α1-adrenergic receptors were reported to be involved in proliferation of hepatocytes *in vitro* during primary culture (Cruise JL. *et al.,* 1985) or *in vivo* during liver regeneration (Agell N *et al*., 1991). However, in juvenile animals or during the process of liver regeneration which follows partial

hepatectomy β-receptors have also been shown to contribute to the proliferation hepatocytes. β-receptor agonist were also reported to prevent Fas-induced apoptosis and death in mice (Andre C *et al*., 1999) but there is no information whether α-adrenergic signalling modulates the sensitivity of hepatocytes towards Fas-mediated cell death.

To test our hypothesis that ADR signalling, which might be defective in TGase2$^{-/-}$ mice, has a protective role against Fas-mediated apoptosis and death in mice, we investigated whether the impaired ADR signalling might be related to the observed down-regulation of Bcl-x_L in TGase2$^{-/-}$ hepatocytes. Wild-type hepatocytes treated for one week with CEC, an ADR antagonist, indeed expressed lower levels of Bcl-x_L and demonstrated increased sensitivity to Fas-mediated death *in vitro*.

In accordance ADR$^{-/-}$ mice similarly to TGase2$^{-/-}$ mice displayed higher mortality after anti-Fas antibody injection than their WT counterparts. Increased liver damage was confirmed by elevated serum AST and ALT levels and by light microscopic examination of liver sections. Western blot analysis of Bcl-x_L expression revealed that ADR$^{-/-}$ hepatocytes similarly to the TGase2$^{-/-}$ ones also contained less Bcl-x_L. These data underline importance of α_1-b adrenergic signalling in the regulation of hepatocyte cell death.

Following various kinds of liver injury, such as partial hepatectomy or CCl_4 administration, increases in the level of Fas ligand, hepatocyte growth factor (HGF) and norepinephrine were detected within 2 hrs following the treatment (Lindroos PM *et al*., 1991). Besides ligation of the HGF receptor, Fas engagement by Fas ligand was shown also to be required for and accelerate liver regeneration after partial hepatectomy, and this effect was related to a simultaneous inhibition of the Fas-mediated cell death pathway in hepatocytes (Desbarats J and Newell MK, 2000). In this context it is worth noting that HGF protects hepatocytes against Fas-mediated death (Kosai K *et al*., 1998), and here we show that ligation of ADR is also inhibitory. In addition, norepinephrine, acting on ADR, was shown to act as co-mitogen with the HGF (Cruise JL *et al*., 1985). These data imply that adrenergic receptor-driven signal transduction pathways participate in the recovery program of the liver following hepatic injury, by promoting both the HGF- and the Fas ligand-driven proliferation of hepatocytes.

The principal finding of our investigation was that loss of TGase2 has an impact not only on the apoptosis but also on the phagocytotic capacity of macrophages. The phagocytosis of apoptotic cells is very effective in the thymus and consequently, the majority of dead cells is

engulfed by macrophages and normally cannot be seen by flow cytometric analysis. Thus, the apparent increase in the percentage of annexin V^+ cells in the thymus of $TGase2^{-/-}$ mice following apoptosis induction (Fig. 7G) suggested that the phagocytosis of apoptotic cells is defective in these mice. This was further supported by the analysis of tissue sections, which revealed an accumulation of non-phagocyted apoptotic cells (Fig. 7H). The defect in clearance of apoptotic cells was also evident after $PbNO_3$ induced liver hyperplasia (Fig. 8C and D) suggesting that it is not an organ-specific defect. Reduced phagocytosis was restricted only to apoptotic cells since yeast, bacteria or opsonized non-apoptotic thymocytes were taken up effectively by $TGase2^{-/-}$ macrophages (Table 2). Based on a series of experiments, we concluded that impaired phagocytosis might be partially a consequence of improper activation of TGF-β by macrophages the activation of which is required for the macrophage recognition of PS on apoptotic cells (Rose DM *et al*., 1995).

At the beginning, apoptosis and subsequent phagocytosis were considered as two distinct processes. Recent studies in mammals and the nematode *Caenorhabditis elegans* have shed some light on the conserved molecular mechanisms involved in this process and imply the presence of a cross-talk between phagocytes and their prey. A series of results now challenge the traditional view of phagocytes as simply scavengers, 'cleaning up' after apoptosis to prevent inflammatory responses, and hence tissue damage. Instead, they suggest that an intensive cross-talk between phagocytes and apoptotic cells ensures that apoptosis goes fast and apoptotic cells are removed efficiently to prevent any inflammatory reaction. Phagocytes were shown to be active in the induction and/or execution of apoptosis in target cells. When cells become dedicated to die macrophages can promote its apoptosis by releasing FasL (Brown SB and Savill J, 1999), TGF-β or nitric oxide (Duffield JS *et al*., 2000). On the other hand, release of chemotactic factors from the dying cell to the extracellular space is required for the proper phagocytosis e.g.: lysophosphatidylcholine (Lauber K *et al*., 2003) and S19 ribosomal protein released from apoptotic cells were shown as an effective chemotactic factor for monocytes (Horino K *et al*., 1998). Furthermore, upon binding to phagocytes, apoptotic corpses re-program them to shut down proinflammatory cytokine production (Cvetanovic M and Ucker DS, 2004). TGase2 participates on both sides in this cross-talk. Activation of TGF-β (Kojima S *et al*., 1993), synthesis of NO (Park KC *et al*., 2004) and formation of the cross-linked S19 ribosomal protein (Horino K *et al*., 1998) were all shown to depend on TGase2 enzyme activity. Besides these

roles, TGase2 also seems to participate in the exposure of PS, as our new ongoing experiments demonstrated that in the absence of TGase2 externalization of PS is delayed.

Autoimmunity is the failure of tolerance to self-antigens. Abnormalities in apoptosis and phagocytosis, as presented above can play a role in autoimmune disease in at least three ways: 1) failure to undergo apoptosis could be responsible for the persistence of auto-reactive lymphocytes 2) failure in the uptake of apoptotic cells presenting self-antigens and 3) death receptors and their ligands could be involved in tissue destruction.

Disintegrated, free apoptotic cells releasing their content exacerbate the local inflammatory response and trigger further leukocyte influx as demonstrated by the infiltration of blood cells in liver tissue sections of TGase2$^{-/-}$ animals after $PbNO_3$ treatment (Fig. 8D and G). The enhanced inflammatory response in TGase2$^{-/-}$ liver can be partly explained by the reduction in the anti-inflammatory TGF-β activity (Christ M *et al*., 1994). As a consequence of impaired phagocytosis, free apoptotic corpses accumulated in the thymus and liver of TGase2$^{-/-}$ animals. Moreover, apoptotic cells express certain nuclear antigens on blebs and other surface structures, rendering these normally sequestered nuclear antigens accessible to the adaptive immune system with potentially harmful consequences. Systemic exposure of the immune system to apoptotic thymocytes in mice results in the transient production of autoantibodies (Rosen A and Casciola-Rosen L, 1999; Mevorach D *et al*., 1998), and that defects in clearance of dead cells are associated with autoimmunity (Pittoni V and Valesini G, 2002). Indeed, the long-term consequence of the defect in TGase2 function was the development of autoimmunity characterized by the appearance of anti-nuclear, anti-cytoskeletal anti-smooth muscle antibodies. Besides the auto-antibodies, increased titers of IgG type anti-IgG2a antibodies and increased number of CD4^{+} and B cells were also detected TGase2$^{-/-}$ animals. This was accompanied by splenomegaly and glomerulonephritis (Fig. 12B and C). Since, in our model the apoptosis of thymocytes is also affected, autoreactive T cells may also accumulate due to the ineffective negative selection and may contribute to the development of autoimmunity. In support of this, we found IgG type anti-IgG antibodies in these mice, the production of which requires the function of autoreactive CD4^{+} T cells.

Taken together, our data suggest that tissue transglutaminase contributes to the proper phagocytosis and to prevention of autoimmunity by participating at multiple levels in the interplay between the dying cells and the phagocytes. First, upregulation of TGase2 promotes the

death of the targets directly by sensitizing the cells toward death stimuli and by enhancing the rate of apoptosis. Second, during apoptosis, TGase2 facilitates the externalization of PS on the cell surface and plays a role in the attraction of macrophages by forming the chemotactic factor cross-linked S19 ribosomal protein. Third, recognition of PS by macrophages triggers the TGase2-dependent production of active TGF-β, which further enhances the death of target cells by enhancing TGase2 expression; suppresses inflammation by down-regulating pro-inflammatory cytokine production of macrophages and stimulates the rate of phagocytosis thereby removing apoptotic cells as potential susceptibility and initiating factors in systemic autoimmunity. Overall, our results provide further support for a cross-talk between cells programmed for death and recruited macrophages and demonstrate that TGase2 is an element of this apopto-phagocytotic machinery, the function of which is critical to prevent inflammation and autoimmunity.

Summary

We investigated the impact of loss of TGase2 on apoptosis program and phagocytotic capacity of macrophages.

Our results demonstrate that TGF-β released by macrophages ingesting apoptotic cells promotes death of thymocytes and since TGase2 is required for TGF-β activation that in the absence of TGase2 *in vivo* apoptosis of thymocytes is delayed. Alteration of the rate of cell death was not restricted to the thymus as livers from TGase2$^{-/-}$ mice showed increased damage accompanied with enhanced inflammatory response compared to WT ones following engagement with agonist anti-Fas antibody as demonstrated by histological examination of liver sections and elevated serum transaminase levels. Increased Fas-sensitivity was correlated with decreased Bcl-x_L levels in TGase2$^{-/-}$ hepatocytes. The similar behaviour of a_1-b-adrenoceptor deficient mice proved that TGase2 as $G_{alpha(h)}$ protein in a_1-b-adrenergic signalling protects hepatocytes against Fas-mediated apoptosis. The principal finding of our investigation was that loss of TGase2 has an impact not only on the apoptosis but also on the phagocytotic capacity of macrophages. We observed reduced phagocytosis of apoptotic cells both *in vitro* and *in vivo* in thymus and liver of TGase2$^{-/-}$ mice. Based on a series of experiments, we concluded that impaired phagocytosis might be partially a consequence of improper activation of TGF-β by macrophages the activation of which is required for the macrophage recognition of PS on apoptotic cells. TGase2$^{-/-}$ mice developed autoimmunity at old age as the consequence of improper removal of apoptotic cells.

Taken together the data suggest that the interplay is initiated between the apoptotic cells and macrophages by the recognition of PS in the outer lipid layer of apoptotic cells by macrophages. This induces an apoptosis-specific form of macrophage activation that involves a TGase2-dependent step in the production of active TGF-β. Activated TGF-β then down-regulates the inflammatory cytokine formation of macrophages and, as it is shown in this work, stimulates the rate of phagocytosis. In addition, TGF-β promotes thymocyte apoptosis and the concurrent accumulation of TGase2 in thymocytes during the apoptosis program *in vivo*. Overall, our results provide further support for a crosstalk between cells programmed for death and recruited macrophages and demonstrate that TGase2 is an element of this apopto-phagocytotic machinery, the function of which is critical to prevent inflammation and autoimmunity.

Part II

Prologue

In the two-year period, when I was working at Karolinska Institute, I investigated the physiology of oral keratinocytes. As part of my training, I acquired knowledge about oligonucleotid microarray technique and established the genetic profile of primary and transformed human buccal keratinocytes. We have found 2534 transcripts showing altered expression compared to normal cells based on a two-fold change criteria (Fig. 25).

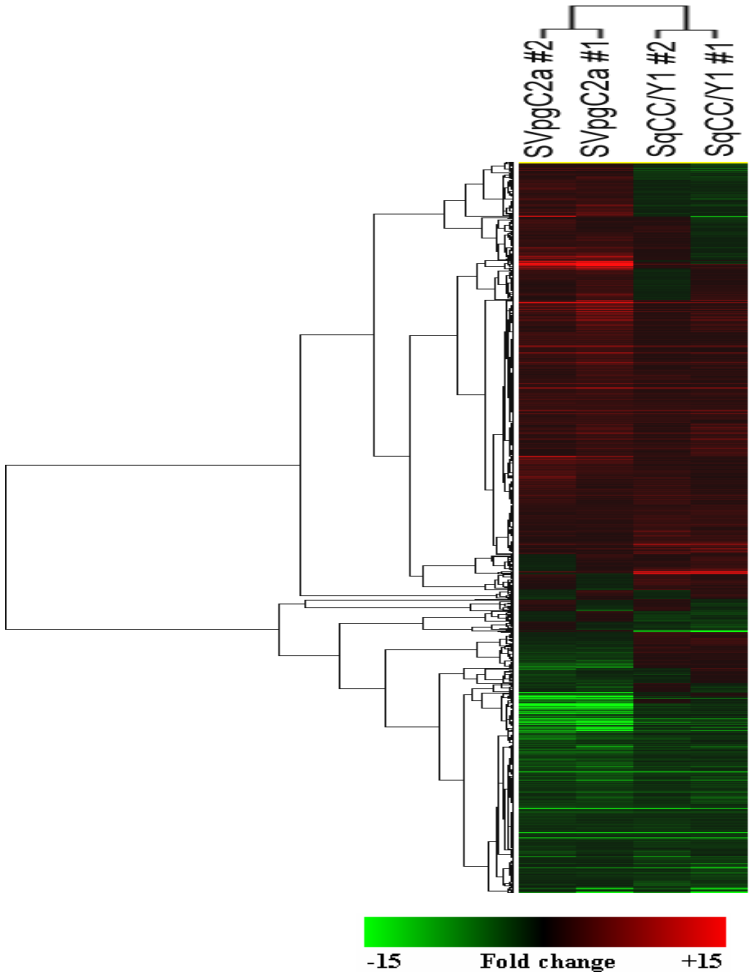

Figure 25 Hierarchical clustering of 2534 transcripts in duplicates of immortalized (SVpgC2a) and malignant (SqCC/Y1) oral keratinocytes samples showing altered expression compared to normal cells based on at least 2-fold fold change value

Parts of these data have been published in different papers dealing with enzymes involved in the metabolism of carbonyls, reactive oxygen and xenobiotics (Hedberg JJ *et al.,* 2001; Vondracek M. *et al.,* 2002), retinoid-related functions (Dressler D *et al*., 2002), expression of different keratins (Hansson A *et al*., 2003) and extracellular matrix related proteins (Sarang Z *et al*., 2003). From these papers, the latter one will be shown in details on the next pages.

.

Introduction

Structure and role of extracellular matrix proteins

The insoluble non-cellular material present between cells throughout the body of multicellular organisms is known as the extracellular matrix (ECM). It is composed out of a complex mixture of secreted molecules, which can be grouped in three major classes: 1. structural proteins providing structural support and tensile strength (collagen and elastin); 2. specialized proteins mainly maintaining cell adhesion and migration (e.g. fibrillin, fibronectin, and laminin); 3. proteoglycans (molecules with protein core to which is attached long chains of repeating disaccharide units termed of glycosaminoglycans). The collagens are a family of fibrous proteins, consisting of at least 20 members, found in all multicellular animals, and the most abundant are types I, II, and III, found in fibrils with a characteristic 67-nm axial period. They are major structural proteins in the extracellular matrix, making up about one-third of protein mass in higher animals. All collagens have a distinctive molecular conformation: a triple-helix composed of three super coiled polyproline II-like helical chains. After collagens are secreted in the form of propeptides, they are converted to collagen molecules by specific proteolytic enzymes, which reside outside the cells. These basic structures organize the extracellular matrix and give it resilience (Persikov AV and Brodsky B, 2002). Fibronectin was the first well-characterized adhesive protein. Fibronectins are dimers of two similar peptides and contain at least six tightly folded domains each with a high affinity for a different substrate such as heparan sulfate, collagen, fibrin and cell-surface receptors such as integrins. The cell-surface receptor-binding domain contains a consensus amino acid sequence, RGDs. At least 20 different fibronectin chains have been identified that arise by alternative RNA splicing of the primary transcript from a single fibronectin gene. Integrins are the major metazoan receptors for cell adhesion to extracellular matrix proteins and, in vertebrates, also play important roles in certain cell-cell adhesions. In addition to mediating cell adhesion, integrins make transmembrane connections to the cytoskeleton and upon ligand binding activate many intracellular signalling pathways that serve to modulate many aspects of cell behavior including proliferation, survival/apoptosis, shape, polarity, motility and differentiation. Functional integrins consist of two transmembrane glycoprotein subunits, called alpha and beta that are non-covalently bound. Both of the subunits contribute to the binding of ligand. Until now 18 alpha and 8 beta subunits have been identified.

From these subunits, at least 25 distinct integrins are formed in nature, which implicates that not all possible combinations exist. Integrins can bind an array of ligands. Common ligands are for example fibronectin and collagen, which are both part of the extracellular matrix (van der Flier A and Sonnenberg A, 2001).

Carcinogenesis in oral epithelia

Development of cancer is generally considered a multistep process. Accordingly, a normal cell may undergo initiation followed by promotion, conversion and progression into a malignant phenotype. (Fig. 26) Oral cancer represents an accumulation of defects in the genes that encode key proteins associated with growth and development. Dysregulation of these proteins is central to malignant conversion. This appears to involve three major changes in cell function: 1. altered cell growth, death and longevity; 2. unencumbered cell movement; and 3. development of a new blood supply (angiogenesis). Specific genes, such as p53, p27, p16, and cyclin D-1, are altered in oral cancer through mutation, amplification, or deactivation. These genes are also frequently altered in many other malignancies. In oral mucosa, etiologic agents -especially tobacco and alcohol, and possibly some viruses- are known to induce alterations in the genes and gene functions associated with cell cycle regulation, contributing to the development of squamous cell carcinoma and epithelial dysplasias.

The development of cancer and the *in vitro* transformation of keratinocytes commonly involves alterations in cell–matrix interactions (Sanders RJ *et al*., 1998). The expression of β1 integrin (ITGB1) subunits and ITGA6/ITGB4 is decreased or lost, whereas that of ITGAV/ITGB6 is commonly increased, in oral squamous cell carcinomas (SCCs) (Thomas GJ *et al*., 1997). Fibronectin and collagens are variably expressed in oral SCCs, affecting the expression of differentiated features and invasion. Moreover, changes in integrin ligands appear to be essential for transforming a pre-malignant epithelium into a malignant lesion (Hornung J *et al*., 1987).

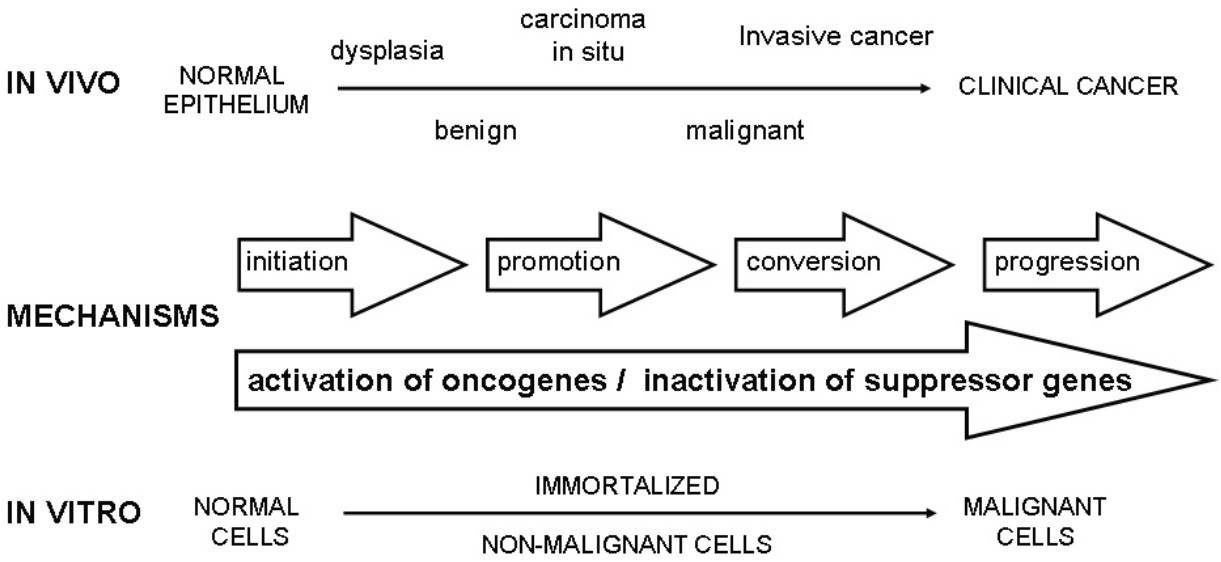

Figure 26 Multistep model of carcinogenesis

The use of cultured human cells provides a valuable tool for studying both normal cellular processes and cancer development *in vitro*. Transformed cell lines are often used as more easily grown substitutes for normal cells, yet immortalized and malignant cells have the potential in providing well-characterized models of early or advanced stages of neoplastic transformation The application of serum-free conditions has permitted the establishment of replicative cultures of normal oral keratinocytes, and the growth of both immortalized and malignant oral keratinocyte lines. Normal (NOK), SV40 T antigen-immortalized (SVpgC2a) and malignant (SqCC/Y1) human buccal keratinocytes model oral cancer development on the basis that they reflect the eventual acquisition of immortality, loss of p53 tumor suppressor function and gain of a tumorigenic phenotype. Accordingly, organotypic epithelia regenerated from NOK, SVpgC2a and SqCC/Y1 demonstrated keratin expression patterns similar to oral normal tissue, dysplastic epithelium and well-differentiated squamous cell carcinoma, respectively (Hansson A. *et al.,* 2001; Hansson A. *et al.,* 2003). Furthermore, SVpgC2a showed elevated proliferation and apoptosis similarly to oral preneoplastic lesions (Hansson A. *et al.,* 2003). In contrast, recent studies in SVpgC2a and SqCC/Y1 indicated preserved expression of certain xenobiotic metabolizing enzymes and ability to form basal lamina proteins relative to NOK (Hansson A *et al.,* 2003; Hedberg JJ *et al.,* 2001; Vondracek M. *et al.,* 2002). Thus, as for development of squamous cell carcinoma, certain cellular functions seem to be fully preserved yet others

substantially altered in this two-step *in vitro* model of carcinogenesis. However, it should be noted that the SqCC/Y1 line were not generated from the immortalized cells and therefore this model represents a discontinuous three-stage carcinogenesis system.

Aim of the study

The aim of the second part of this work was to investigate the expression of ECM-related proteins and their effect on the growth and migration of human oral keratinocytes.

The specific sub-aims were to:

- to determine the expression levels of fibronectin, collagens and integrines in normal, SV40 T transformed and malignant human oral keratinocytes using Affymetrix oligonucleotid microarray.
- to investigate the effect of extracellular fibronectin and collagen on the growth, colony-forming efficiency and migration of normal, SV40 T transformed and malignant human oral keratinocytes.

Materials and Methods

Cell culture procedures. For the primary cell cultures, clinically healthy human buccal tissue (used with the approval of the Karolinska Institute ethical committee) was incubated for 18–24 hours at 4°C in phosphate-buffered saline (PBS) containing 0.17% trypsin. The mixture of single cells and small aggregates formed was resuspended in serum-free growth medium (EMHA; an epithelial medium with high levels of amino acids denoted as EMA was recently re-termed EMHA) and plated onto fibronectin/collagen (FN/COL)-coated dishes at a density of $5x10^3$ cells/cm^2. Coated dishes were prepared by incubating a mixture of 10 μg/ml FN (Sigma Chemical Co., St. Louis, MO), 30 μg/ml Vitrogen (contains bovine dermal collagen of primarily type 1) (Cohesion Technologies Inc., Palo Alto, CA) and 10 μg/ml bovine serum albumin (Sigma Chemical Co.) dissolved in phosphate-buffered saline on tissue culture plastic (Falcon, Becton Dickinson & Co**,** Franklin Lakes, NJ) for 1 h. The immortal cell line SVpgC2a, derived by transfection and stable integration of the SV40T antigen into buccal keratinocytes (Kulkarni PS *et al*., 1995), and the buccal carcinoma cell line SqCC/Y1 were also cultured in serum-free EMHA. Cell growth assessments in mass culture and clonal density used NOK lines obtained from different donors in passage 2, the SVpgC2a line in passages 59-64, and the SqCC/Y1 line in passages 115-120. For assessment of the influences of presence or absence of FN/COL-coating in the respective cell types, cells were transferred in 60 or 100 mm dishes at 4.2 to $6.0x10^3$ cells/cm^2 at least twice over minimally a two-week period with or without the coating mixture, respectively, before initiation of the actual experiments. The micro-array analysis used cultures of NOK in passage 2, SVpgC2a in passages 63 and 64, and SqCC/Y1 in passages 125 and 128 utilizing cells grown without FN/COL-coating.

Preparation of labelled cRNA. Methods for cRNA preparation, the hybridization reactions and data analysis were provided by the manufacturer (Affymetrix™). Briefly, total RNA was prepared with RNeasy (Qiagen) from 3 x 10^6 cells of each type. Double-stranded cDNA was synthesized from 25 μg total RNA using a cDNA synthesis kit (SuperScript Choice system; GibcoBRL Life Technologies). Labelled cRNA was *in vitro* transcribed with 1.5 μg cDNA as template incorporating biotinylated CTP and UTP (Enzo RNA Transcript Labelling Kit; Enzo Diagnostics). The cRNA was purified with RNeasy affinity columns (Qiagen) and subsequently

fragmented in 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate at 94°C for 35 min.

Array hybridization. To determine gene expressions, microarray chips with oligonucleotides corresponding to over 12000 human transcripts (HG-U95A, Affymetrix, Santa Clara, CA, USA) were hybridized for 16 h at 45°C with 10µg of labelled cRNA from NOK, SVpgC2a and SqCC/Y1 cultures, respectively. Following hybridization the arrays were washed under stringent conditions, stained with streptavidin-phycoerythrin (Molecular Probes Europe BV, Leiden, Netherlands), washed again and subsequently scanned at 570nm using a Hewlett Packard gene array scanner. Microarray data were analyzed with GeneChip®4.0 software (Affymetrix™), then imported and sorted in Microsoft Excel and Cluster software, and then visualized with TreeView (downloaded from http://www.rana.lbl.gov). All chips were scaled to a target intensity of 250 for inter-array comparisons. The presence of individual genes was described by the *absolute call* (based on *Positive/Negative Ratio*, *Positive Fraction* and *Log Average Ratio*), while changes in expression levels were quantified by the *average difference (intensity)* and *fold change* values. (for details see GeneChip®4.0 Expression Analysis Algorithm Tutorial). Gene expression is generally considered to differ among samples based on a two-fold change. The number of transcripts judged as present in NOKs, and SVpgC2a and SqCC/Y1 cells in hybridization 1 and 2 were 3830/3958, 4865/4467 and 3887/4533, respectively.

Cell Growth Assessment in Mass Culture. Cells were inoculated at standard passage densities, i.e., 4.2 to 6.0 x 10^3 cells/cm^2 and incubated for 24 h in EMHA, and medium was thereafter exchanged at second day intervals. Net growth, as indicated by the total cell number, was determined under a Nikon TMS inverted microscope, using a special 10x ocular equipped with a check-pattern of 1 mm^2 in size. At the indicated time points, the cultures were separately removed from the incubator and kept warm with a heated fan while under the microscope for the short duration of the counting procedure. The mean number of cells per mm^2 was calculated in 10 randomly selected fields, and the value multiplied with the total area of the dish to obtain the total cell number. Notably, from the aspect of time, each culture (dish) served as its own control.

Colony-forming Efficiency(CFE). The CFE of each cell type was assayed as previously described (Nilsson JA *et al*., 1998). Briefly, the cells were seeded at a density of 50 cell/cm^2 in 60 mm dishes, and following change of medium at 24 h, the cells were incubated for another 7 days in EMHA with one intermediate medium change. The cultures were then fixed in 10% formalin

and stained with 1% aqueous crystal violet. The mean CFE was determined from duplicate dishes and based on microscopic counting of the colonies. Based on the average cell division rate of at least 0.5 populations doubling per day, individual cell clones were considered a colony when containing at least 12 cells.

Results

Oligonucleotide microarray. A set of microarrays with oligonucleotides corresponding to over 12000 human genes in duplicate was hybridized with cRNA prepared from normal (NOK), SV40T-immortalized (SVpgC2a) and malignant (SqCC/Y1) oral keratinocytes to determine presence/absence and the expression level of fibronectin, collagens and integrins (Fig. 27). Probes for fibronectin, 29 different collagen chains and 24 different integrin subunits were present on the chip, including more that one probe for some of the genes. The expression profiles were presented as transcripts for which differences were observed among the cell lines (Fig. 27A) and transcripts that did not differ among the cell lines (Fig. 27B). Fibronectin transcripts were abundant in all cell lines, whereas the levels of collagen chains and integrin subunits varied. For collagen chains, the number of transcripts detected as present, marginally present or absent was 8/0/29 in the two NOK lines, 9-14/1-2/22-26 in SvpgC2a and 8-9/0-1/28 in SqCC/Y1, respectively. For integrin subunits, the number of transcripts detected as present, marginally present or absent was 15-16/0-2/21-22 in the NOK lines, 12-13/0-1/24-26 in SvpgC2a and 13/0-1/24-25 in SqCC/Y1, respectively. Overall, transcripts for 20 genes (11 collagen chains and 9 integrin units) showed altered expression level, while 18 collagens and 15 integrins were detected as absent or expressed at the same level in SVpgC2a and/or SqCC/Y1 compared to the NOK (Fig. 27B).

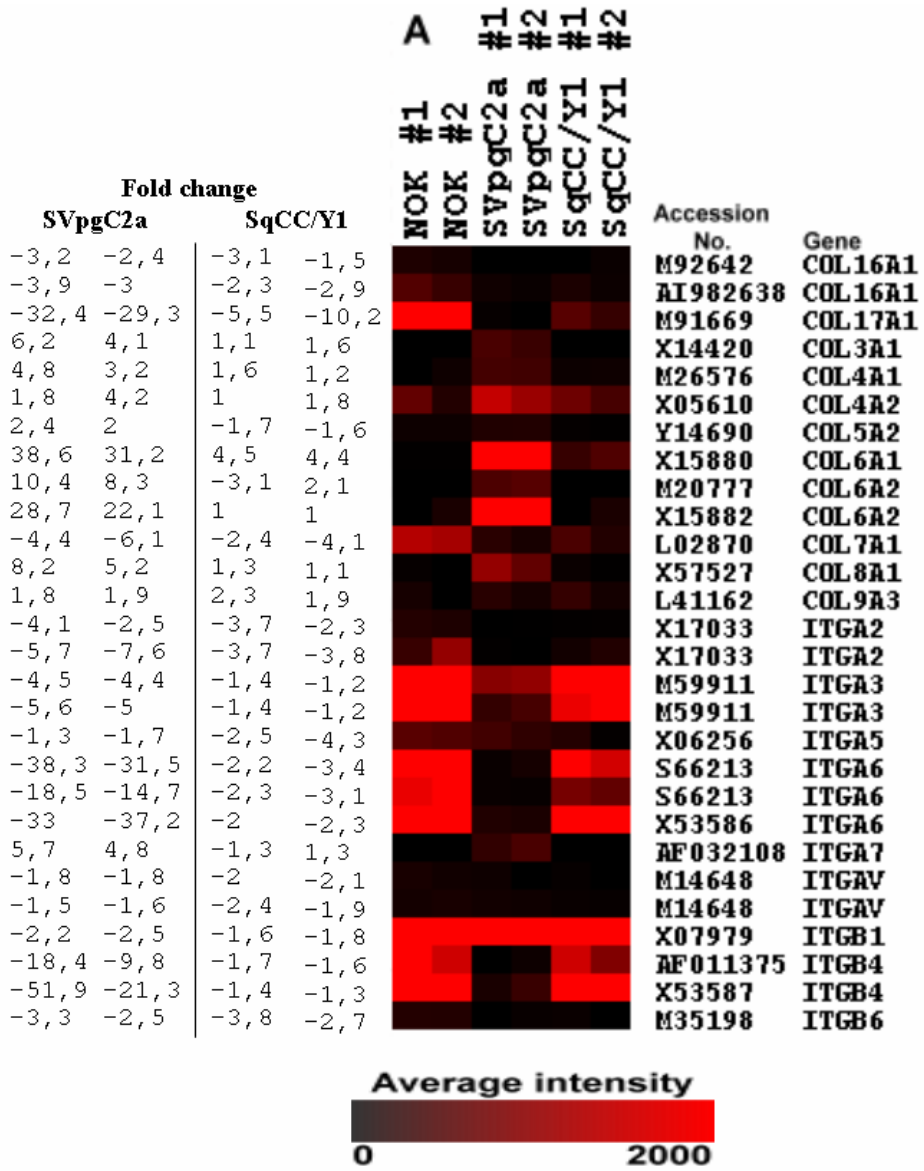

Figure 27A Transcripts that differed among cell lines (based on a minimum of a two-fold difference in gene expression, as calculated by the GeneChip 4.0 software). The colour code used to express the "average intensity", reflecting intensity of expression, is shown at the bottom. Abbreviations relative to the "absolute call": A = absent; M = marginally present; P = present. COL = collagen; FN = fibronectin; ITG = integrin

.

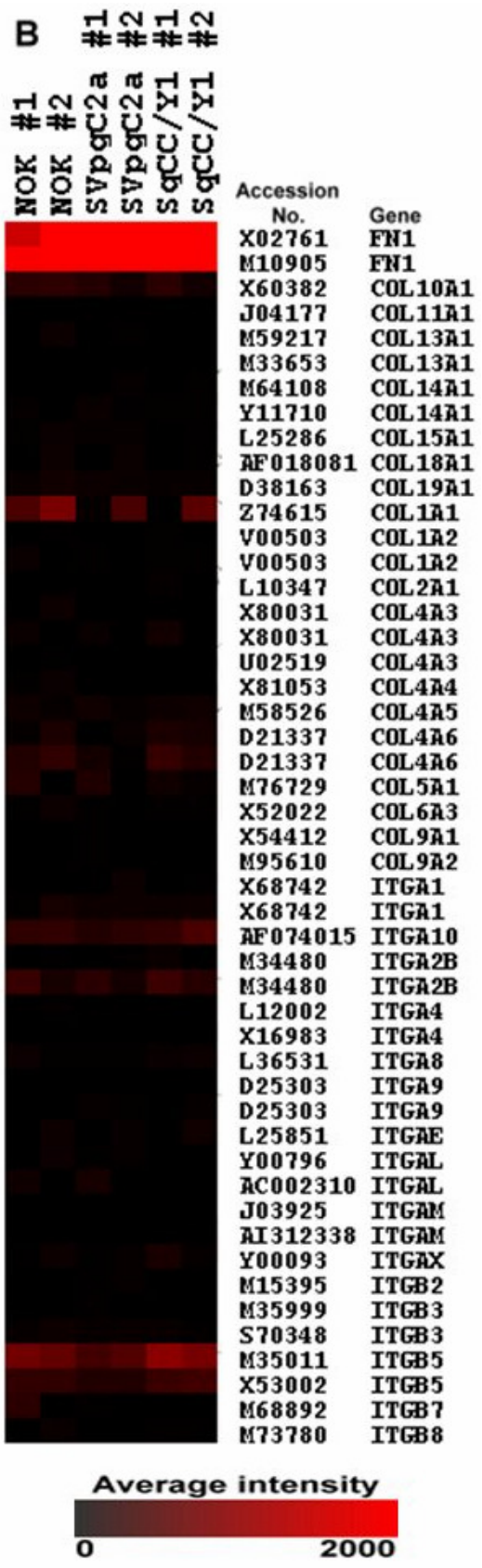

Figure 27B Transcripts that did not differ among cell lines (based on a minimum of a two-fold difference in gene expression, as calculated by the GeneChip® 4.0 software). The colour code used to express the "average intensity", reflecting intensity of expression, is shown at the bottom. Abbreviations relative to the "absolute call": A = absent; M = marginally present; P = present. COL = collagen; FN = fibronectin; ITG = integrin.

Effect of fibronectin-collagen coating on cell growth. The growth of the respective cell lines from a sparse to a confluent state on non-coated and coated dishes was initially assessed. With the selected seeding densities, the cell lines became confluent at 5 days. The growth rates of the cultures were greater at the terminal phase of the experiments, i.e. at higher cell density. Relative to NOKs, higher cell numbers per culture dish were obtained for the SVpgC2a and SqCC/Y1 cells. For all the cell types, small but non-significant variations in cell numbers were seen, with or without coating with FN–COL (Fig. 28).

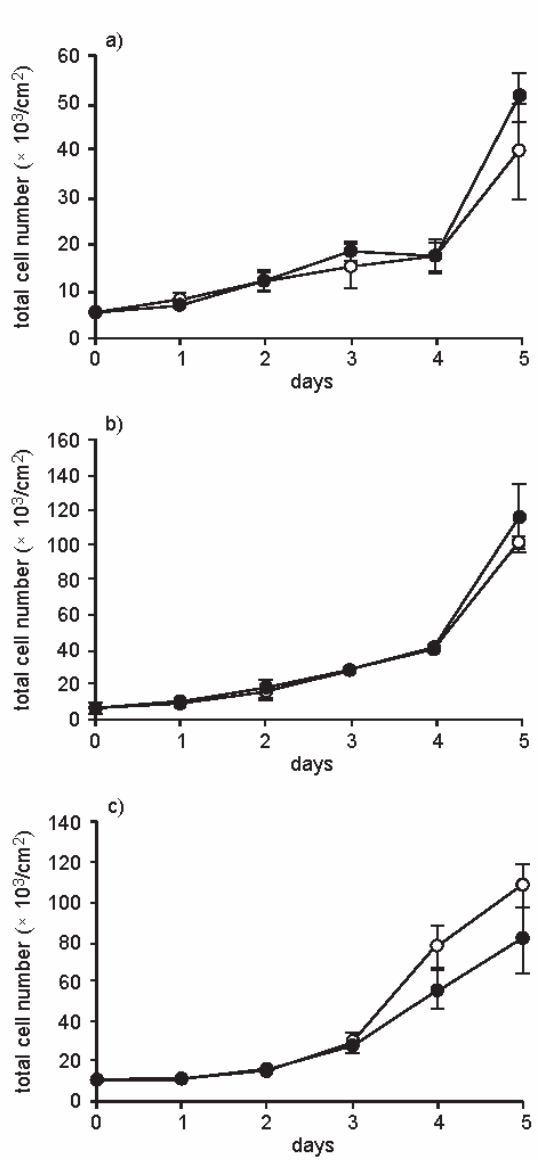

Figure 28 Growth of a) NOKs, b) SVpgC2a cells and c) SqCC/Y1 cells in the presence (open circle) or absence (filled circle) of FN–COL. The cell lines were inoculated at the indicated densities with or without FN–COL, and the

net growth rate was assessed daily under phase contrast microscopy until the cultures became confluent. Mean ± SEM values from three separate experiments are shown.

Effect of fibronectin-collagen coating on cell CFE. The CFE of each of the cell lines was then assessed. The CFE of SVpgC2a cells was two-fold higher than the CFEs of NOKs and SqCC/Y1 cells. The CFE value for each cell line was slightly higher without FN–COL coating; however, the differences were not statistically significant (Fig. 29).

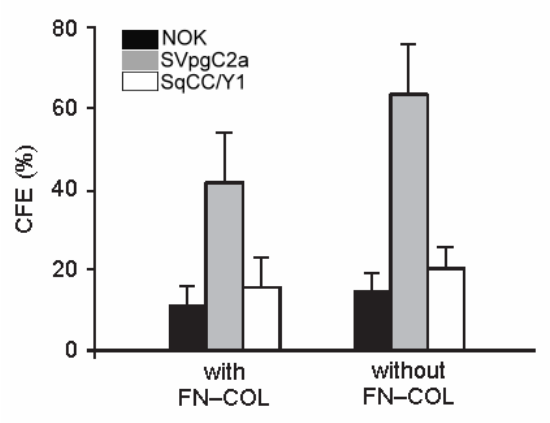

Figure 29 Effect of FN-COL coating on CFE. Cells were cultured with or without FN–COL, as indicated. The CFE was determined from sparsely seeded cells that were then cultured until clones could be visualised under phase contrast microscopy. Clones consisting of 12 or more cells were scored as colonies. Mean ± SEM values from three separate experiments are shown.

Effect of fibronectin-collagen coating on cell migration. Based on the known stimulatory action of FN–COL on normal keratinocyte migration (O'Toole EA, 2001), typical colony morphologies of NOKs and SVpgC2a and SqCC/Y1 cells were investigated. The clones/colonies from all the cell lines were reproducibly more dispersed on FN–COL-coated surfaces, with a larger surface area per colony (Fig. 30). This effect of FN–COL was consistently seen in all the NOK, SVpgC2a and SqCC/Y1 cultures tested.

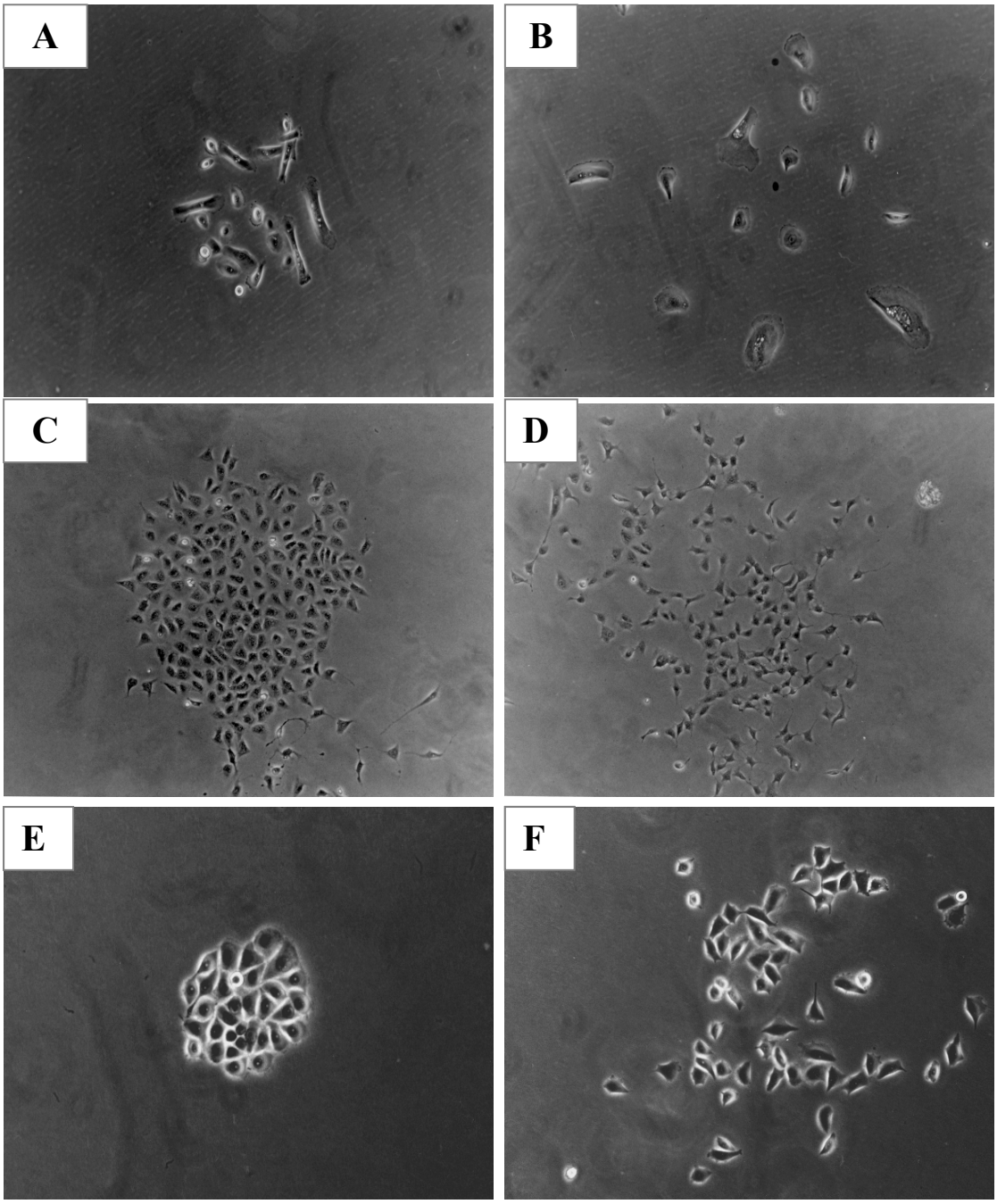

Figure 30 Colony morphologies of normal (NOK), SV40 T-antigen-immortalised (SVpgC2a) and malignant (SqCC/Y1) human oral keratinocyte lines grown with or without an extracellular human fibronectin–bovine collagen type I (FN–COL) matrix Without FN–COL: a) NOKs; c) SVpgC2A cells and e) SqCC/Y1 cells. With FN–COL: b) NOKs; d) SVpgC2A cells and f) SqCC/Y1 cells. Cells were cultured as described in the legend to Figure 3. Typical morphologies indicate increased cell migration in the presence of FN–COL.

Discussion

The reduction and replacement of animal experimentation by *in vitro* methods, including the use of various cell lines to model multistep carcinogenesis, is likely to be facilitated and made more reliable by the standardisation of culture procedures. Various studies have addressed the requirement for, and expression of, ECM proteins (for example, fibronectin and collagen), and ECM protein receptors (for example, integrins), in normal human keratinocyte lines. However, few studies have assessed these aspects in transformed keratinocyte lines. We used the oral keratinocyte lines NOK, SVpgC2a and SqCC/Y1 under standardised, serum-free culture conditions, based on the concept that they might represent a model for progression from normality to malignancy (Grafstrom RC *et al*., 1997). Gene expression profiles of fibronectin, collagens and integrins were determined, and the responsiveness of the cell lines to FN–COL, in terms of growth, cloning efficiency and migration was investigated. The composite analysis supported the synthesis and functional interaction of several ECM-related components in oral keratinocytes, although notable differences in gene expression and growth characteristics were observed among the cell lines. With regard to increased expression of collagens and decreased expression of integrins, the immortalised, non-malignant state of SVpgC2a differed to a greater degree from NOKs than the malignant state of SqCC/Y1. Enhanced cell migration in response to FN–COL coating was clearly indicated in all the cell lines, but without a simultaneous influence on cell growth. The results show that migration and proliferation can occur as separate events in these keratinocyte lines, and that, for the purpose of routine cell line expansion, the standardised transfer protocols could be simplified by using regular, non-coated tissue culture dishes. Oral SCC development may involve significant changes in the expression of integrins and ECM proteins (Thomas GJ and Speight PM, 2001). Application of the Affymetrix technique demonstrated the presence of transcripts for such proteins in NOKs, and in SVpgC2a and SqCC/Y1 cells (Fig. 1). The expression of collagen chains in SqCC/Y1 cells, which were derived from a well-differentiated buccal SCC (Pitman SW *et al*., 1983), was similar to that in NOKs, although decreased levels of types COL7A1 and COL17A1 and increased level of COL6A1 were observed. SCC may arise as a complication of inherited epidermolysis bullosa, a disease that is characterised by the functional loss of these collagens (Mallipeddi R, 2002). Loss of COL7A1 is common in highly differentiated SCCs and may serve as a marker for invasiveness; SqCC/Y1

cells can also grow invasively in organotypic culture (Hansson A *et al*., 2001). From a qualitative standpoint, the expression of integrin subunit transcripts in NOKs was similar to the expression patterns obtained in various *in vivo* and *in vitro* studies of oral epithelium (Thomas GJ and Speight PM, 2001). Integrin expression is commonly altered in oral SCC, although primarily in poorly differentiated lesions (Thomas GJ, Jones J, Speight PM, 1997). Accordingly, the integrin expression pattern in SqCC/Y1 cells was similar to that in NOKs, without the alterations that typify poorly differentiated tumours. No or reduced levels of several integrin subunits were observed in SVpgC2a cells for example, ITGA2, ITGA3, ITGA6 and ITGB1, and an increased expression of several collagen transcripts. The experienced low levels of $\alpha 3\beta 1$ RNA in SVpgC2a cells compared to NOK and SqCC/Y1 was similar to those found in premalignant gingival keratinocytes cells and SCCs (Ghosh S *et al*., 2002) where it had been shown to regulate the expression of an invasiveness-related proteinase, urinary-type plasminogen activator. Gain of ITGA7 and several collagen chains suggests that the SVpgC2a cells might have acquired selected characteristics of non-epithelial cells (Ziober BL and Kramer RH, 1996). These alterations may contribute to the poorly differentiated, basaloid phenotype of SVpgC2a cells (Hansson A *et al*., 2001), and also to the low coherence among cells in regenerated epithelia and the loose attachment of monolayers to tissue culture plastic (Grafström RC, unpublished observations). The detection of abundant fibronectin transcripts in all the cell lines tested agreed with previous studies on normal and transformed epidermal keratinocyte lines (Brown KW and Parkinson EK, 1985). The microarray results obtained in this study will require confirmation, for example by quantitative reverse transcriptase-polymerase chain reaction, or at the protein level. However, where reference could be made, the transcript assessments agreed with the published literature; for example, the expression of integrin proteins in oral epithelium (Thomas GJ and Speight PM, 2001). Keratinocyte proliferation is often coupled with migration, but can occur independently (Woodley DT *et al*., 1993). Previous work has shown that FN–COL coating can increase the primary culture yield of NOKs, involving increased CFE, growth rates and migration (Sundqvist K *et al*., 1991). As demonstrated here, transferred NOK, SVpgC2a and SqCC/Y1 cultures exhibited enhanced migration on FN–COL, without significant alterations in growth or CFE. Growth assessments reflect broad biological endpoints, involving the net outcome of proliferation versus terminal differentiation and apoptosis, and many cell functions are thus likely to be expressed similarly with or without FN–COL. The growth stimulatory effect of FN–COL on

primary cultures may relate to the fact that ECM proteins can support attachment and block suspension-induced terminal differentiation (Adams JC, Watt FM, 1989). In contrast, the activated synthesis of ECM ligands and receptors may make the transfer of cell lines independent of FN–COL coating. The consistent migratory stimulus of FN–COL observed here supports findings that different integrins may collaborate and substitute for one another, with regard to various physiological effects (Koivisto L *et al*., 1999). In conclusion, the expression and function of fibronectin, collagens and integrin receptors were analysed in a three-stage model of tumour progression, by the application of microarray analysis and growth assays. Transcripts of fibronectin, and various collagens and integrin receptors, the latter recognising fibronectin and/or collagens as ligands, were detected. The transcript profiles obtained reflect the multiple changes in ECM–cell interactions observed during oral carcinogenesis, including a shift from the synthesis of integrins to collagens in the immortalised, non-malignant SVpgC2a cells. Accordingly, the loss of integrins can be associated with a loss of differentiation without malignancy. The cell types tested, except for the migration, behaved similarly in the absence or presence of an ECM consisting of FN–COL, albeit with different growth characteristics and gene expression profiles, implying that the transfer of keratinocyte lines may be simplified by the omission of FN–COL. Studies of other integrin-binding ECM molecules may also reveal hidden consequences of the different expression levels detected in the cell lines. The future assessment of immortalised cell lines, such as SVpgC2a, may provide insight into the early stages of cancer progression and stimulate a search for identical changes in preneoplasia. As demonstrated here, the *in vitro* modelling of different phases of cancer development involving several cell lines may be possible, under highly defined conditions.

Summary

In this study we investigated the requirement for, and expression of, ECM proteins (fibronectin and collagen), and ECM protein receptors (integrins) for normal, immortalised and malignant oral keratinocytes growth and migration.

We found that with regard to increased expression of collagens and decreased expression of integrins, the immortalised, non-malignant state of SVpgC2a differed to a greater degree from NOKs than the malignant state of SqCC/Y1. We also found that SVpgC2a cells had the highest growth rate and clonogenic capacity assessed by CFE assays. The growth and CFE of any of the cell types were not influenced by the absence or presence of ECM molecules. However we observed enhanced migration of each cell types in the presence of FN-COL suggesting that different integrins may collaborate and substitute for one another, with regard to various physiological effects.

The cell lines tested responded similarly to an ECM consisting of FN–COL, albeit with different growth characteristics and gene expression profiles, implying that the transfer of keratinocyte lines may be simplified by the omission of FN–COL. The future assessment of immortalised cell lines, such as SVpgC2a, may provide insight into the early stages of cancer progression and stimulate a search for identical changes in preneoplasia.

Acknowledgements

I wish to thank my supervisors Zsuzsa Szondy and Roland Grafstrom for helping and guiding me in my work.

I would like to thank Prof. Laszlo Fesus, head of the Department of Biochemistry and Molecular Biology for letting me work in his department.

I also would like to thank our collaborators for their excellent work in my experiments.

Many thanks to all the people in the department, and special thanks to the 104 laboratory, for their support.

Finally, I thank Eva, my girlfriend, for encouraging me and caring for me.

References

Achyuthan KE, Greenberg CS (1987) Identification of a guanosine triphosphate-binding site on guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity. *Journal of Biological Chemistry* 262(4):1901-6

Adams JC, Watt FM (1989) Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature. 340(6231):307-9

Aeschlimann D, Thomazy V. (2000) Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases. *Connective Tissue Research* 41(1):1-27

Agell N, Pujol MJ, Rius E, Bachs O (1991) Regulation of DNA polymerase alpha activity by the alpha 1-adrenergic receptors in proliferatively activated rat liver cells. *Biochemical and Biophysical Research Communications* 177(3):973-8

Akimov SS, Belkin AM. (2001) Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. *Blood* 98(5):1567-76

Andre C, Couton D, Gaston J, Erraji L, Renia L, Varlet P, Briand P, Guillet JG (1999) beta2-adrenergic receptor-selective agonist clenbuterol prevents Fas-induced liver apoptosis and death in mice. *American Journal of Physiology* 276(3 Pt 1):G647-54

Antonyak MA, Singh US, Lee DA, Boehm JE, Combs C, Zgola MM, Page RL, Cerione RA (2001) Effects of tissue transglutaminase on retinoic acid-induced cellular differentiation and protection against apoptosis. *Journal of Biological Chemistry* 276(36):33582-7

Banhegyi G, Fulceri R, Bellomo G, Romani A, Pompella A, Benedetti A (1991) Role of nonmitochondrial Ca2+ pool in the synergistic stimulation by cyclic AMP and vasopressin of Ca2+ uptake in isolated rat hepatocytes. *Archives of Biochemistry and Biophysics* 287(2):320-8

Beresford PJ, Zhang D, Oh DY, Fan Z, Greer EL, Russo ML, Jaju M, Lieberman J (2001) Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *Journal of Biological Chemistry* 276(46):43285-93

Bergamini CM (1988) GTP modulates calcium binding and cation-induced conformational changes in erythrocyte transglutaminase. *FEBS Letters* 239(2):255-8

Blaxall HS, Hass NA, Bylund DB (1994) Expression of alpha 2-adrenergic receptor genes in rat tissues. *Receptor* 4(3):191-9

Boehm JE, Singh U, Combs C, Antonyak MA, Cerione RA (2002) Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. *Journal of Biological Chemistry* 277(23):20127-30

Brown KW, Parkinson EK (1985) Alteration of the extracellular matrix of cultured human keratinocytes by transformation and during differentiation. *International Journal of Cancer* 35(6):799-807

Brown SB, Savill J (1999) Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leukocytes. *Journal of Immunology* 162, 480–485

Candi E, Oddi S, Terrinoni A, Paradisi A, Ranalli M, Finazzi-Agro A, Melino G (2001) Transglutaminase 5 cross-links loricrin, involucrin, and small proline-rich proteins in vitro. *Journal of Biological Chemistry* 276(37):35014-23

Cavalli A, Lattion AL, Hummler E, Nenniger M, Pedrazzini T, Aubert JF, Michel MC, Yang M, Lembo G, Vecchione C, Mostardini M, Schmidt A, Beermann F, Cotecchia S. (1997) Decreased blood pressure response in mice deficient of the alpha1b-adrenergic receptor. *Proceedings of the National Academy of Sciences USA* 94(21):11589-94

Chen JS, Mehta K (1999) Tissue transglutaminase: an enzyme with a split personality. *International Journal of Biochemistry & Cell Biology* 31(8):817-36
de la Coste A, Fabre M, McDonell N, Porteu A, Gilgenkrantz H, Perret C, Kahn A, Mignon A (1999) Differential protective effects of Bcl-xL and Bcl-2 on apoptotic liver injury in transgenic mice. *American Journal of Physiology* 277:G702-8
Christ M, McCartney-Francis NL, Kulkarni AB, Ward JM, Mizel DE, Mackall CL, Gress RE, Hines KL, Tian H, Karlsson S. (1994) Immune dysregulation in TGF-beta 1-deficient mice. *Journal of Immunology* 153(5):1936-46
Cruise JL, Houck KA, Michalopoulos GK. (1985) Induction of DNA synthesis in cultured rat hepatocytes through stimulation of alpha 1 adrenoreceptor by norepinephrine. *Science*. 227(4688):749-51
Cvetanovic M, Ucker DS (2004) Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *Journal of Immunology* 172(2):880-9
De Laurenzi V, Melino G (2001) Gene disruption of tissue transglutaminase. *Molecular and Cellular Biology* 21(1):148-55
Desbarats J, Newell MK. (2000) Fas engagement accelerates liver regeneration after partial hepatectomy. *Nature Medicine* 6(8):920-3
Diehl AM, Abdo S, Brown N. (1990) Supplemental putrescine reverses ethanol-associated inhibition of liver regeneration. *Hepatology* 12:633-7.
Dressler D, Sarang Z, Szondy Z, Engelhart K, Grafstrom RC (2002) Expression of retinoid-related genes in serum-free cultures of normal, immortalized and malignant human oral keratinocytes. *International Journal of Oncology* 20(5):897-903
Duffield JS, Erwig LP, Wei X, Liew FY, Rees AJ, Savill JS (2000) Activated macrophages direct apoptosis and suppress mitosis of mesangial cells. *Journal of Immunology* 164(4):2110-9
Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM (1998a) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *Journal of Clinical Investigation* 101(4):890-8
Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992b) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of Immunology* 148(7):2207-16
Fazekas G, Palfi G, Wolff-Winiski B, Rosenwirth B, Dukor P, Gergely J, Rajnavolgyi E (1995) IgG isotype-specific auto-antibodies bind preferentially to cross-linked membrane Ig. *International Immunology* 7(7):1125-34
Fesus L, Madi A, Balajthy Z, Nemes Z, Szondy Z (1996a) Transglutaminase induction by various cell death and apoptosis pathways. *Experientia* 52(10-11):942-9
Fesus L, Piacentini M (2002) Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends in Biochemical Sciences* 27(10):534-9
Fesus L, Thomazy V, Autuori F, Ceru MP, Tarcsa E, Piacentini M (1989b) Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action. *FEBS Letters* 245(1-2):150-4
Fesus L, Thomazy V, Falus A (1987) Induction and activation of tissue transglutaminase during programmed cell death. *FEBS Letters* 224(1):104-8
van der Flier A, Sonnenberg A (2001) Function and interactions of integrins. *Cell & Tissue Research* 305(3):285-98

Ghosh S, Munshi HG, Sen R, Linz-McGillem LA, Goldman RD, Lorch J, Green KJ, Jones JC, Stack MS (2002) Loss of adhesion-regulated proteinase production is correlated with invasive activity in oral squamous cell carcinoma. *Cancer* 95(12):2524-33
Gordon FH, Song L-W, Kumar R, Nanda N, Wu J, Zern MA. (2002) The role of tissue transglutaminase in hepatocyte apoptosis. *Hepatology* 36(2):379A.
Grafstrom RC, Noren UG, Zheng X, Elfwing A, Sundqvist K (1997) Growth and transformation of human oral epithelium in vitro. *Recent Results in Cancer Research* 143:275-306
Greenberg CS, Birckbichler PJ, Rice RH (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB Journal* 5(15):3071-7
Grenard P, Bates MK, Aeschlimann D (2001) Evolution of transglutaminase genes: identification of a transglutaminase gene cluster on human chromosome 15q15. Structure of the gene encoding transglutaminase X and a novel gene family member, transglutaminase Z. *Journal of Biological Chemistry* 276(35):33066-78
Gruol DJ, Altschmied J (1993) Synergistic induction of apoptosis with glucocorticoids and 3',5'-cyclic adenosine monophosphate reveals agonist activity by RU 486. *Molecular Endocrinology* 7(1):104-13
Hansson A, Bloor BK, Haig Y, Morgan PR, Ekstrand J, Grafstrom RC (2001) Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture. *Oral Oncology* 37(5):419-30
Hansson A, Bloor BK, Sarang Z, Haig Y, Morgan PR, Stark HJ, Fusenig NE, Ekstrand J, Grafstrom RC (2003) Analysis of proliferation, apoptosis and keratin expression in cultured normal and immortalized human buccal keratinocytes. *European Journal of Oral Sciences* 111(1):34-41
Hasegawa G, Suwa M, Ichikawa Y, Ohtsuka T, Kumagai S, Kikuchi M, Sato Y, Saito Y (2003) A novel function of tissue-type transglutaminase: protein disulphide isomerase. *Biochemical Journal* 373(Pt 3):793-803
Hedberg JJ, Grafstrom RC, Vondracek M, Sarang Z, Warngard L, Hoog JO (2001) Micro-array chip analysis of carbonyl-metabolising enzymes in normal, immortalised and malignant human oral keratinocytes. *Cellular and Molecular Life Sciences* 58(11):1719-26
Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19(1):245-54
Herrera B, Alvarez AM, Beltran J, Valdes F, Fabregat I, Fernandez M (2004) Resistance to TGF-beta-induced apoptosis in regenerating hepatocytes. *Journal of Cellular Physiology* 201(3):385-92
Hickman JA, Boyle CC (1997) Apoptosis and cytotoxins. *British medical bulletin* 53(3):632-43
Ho KC, Quarmby VE, French FS, Wilson EM (1992) Molecular cloning of rat prostate transglutaminase complementary DNA. The major androgen-regulated protein DP1 of rat dorsal prostate and coagulating gland. *Journal of Biological Chemistry* 267(18):12660-7
Hoeppner DJ, Hengartner MO, Schnabel R (2001) Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans. *Nature* 412(6843):202-6
Horino K, Nishiura H, Ohsako T, Shibuya Y, Hiraoka T, Kitamura N, Yamamoto T (1998) A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells. *Laboratory Investigation* 78(5):603-17
Hornung J, Bohnert A, Phan-Than L, Krieg T, Fusenig NE (1987) Basement membrane formation by malignant mouse keratinocyte cell lines in organotypic culture and transplants:

correlation with degree of morphologic differentiation. *Journal of Cancer Research and Clinical Oncology* 113(4):325-41.
Houck KA, Michalopoulos GK (1989) Altered responses of regenerating hepatocytes to norepinephrine and transforming growth factor type beta *Journal of Cellular Physiology* 141(3):503-9
Huynh ML, Fadok VA, Henson PM (2002) Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *Journal of Clinical Investigations* 109(1):41-50
Iismaa SE, Chung L, Wu MJ, Teller DC, Yee VC, Graham RM (1997) The core domain of the tissue transglutaminase Gh hydrolyzes GTP and ATP. *Biochemistry* 36(39):11655-64
Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* 388(6638):190-5.
Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *British Journal of Cancer* 26: 239-257
Koivisto L, Larjava K, Hakkinen L, Uitto VJ, Heino J, Larjava H (1999) Different integrins mediate cell spreading, haptotaxis and lateral migration of HaCaT keratinocytes on fibronectin. *Cell Adhesion and Communications* 7(3):245-57
Kojima S, Nara K, Rifkin DB (1993) Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells. *Journal of Cellular Biology* 121(2):439-48
Kosai K, Matsumoto K, Nagata S, Tsujimoto Y, Nakamura T (1998) Abrogation of Fas-induced fulminant hepatic failure in mice by hepatocyte growth factor. *Biochemical and Biophysical Research Communications* 244(3):683-90
Krammer PH (1999) CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Advances in Immunology* 71: 163-210
Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S (2001) Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signalling complex. *Journal of Biological Chemistry* 276(23):20633-40
Kulkarni PS, Sundqvist K, Betsholtz C, Höglund P, Wiman KG, Zhivotsky B, Bertolero F, Liu Y, Grafström RC (1995). Characterisation of human buccal epithelial cells transfected with the simian virus 40 T-antigen gene. *Carcinogenesis* 16, 2515–2521.
Kunstle G, Leist M, Uhlig S, Revesz L, Feifel R, MacKenzie A, Wendel A (1997) ICE-protease inhibitors block murine liver injury and apoptosis caused by CD95 or by TNF-alpha. *Immunology Letters* 55(1):5-10
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 277:680-685.
Lassus P, Opitz-Araya X, Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297(5585):1352-4
Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, Xu Y, Autenrieth IB, Schulze-Osthoff K, Belka C, Stuhler G, Wesselborg S (2003) Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* 113(6):717-30
Lecoeur H, Ledru E, Gougeon ML (1998) A cytofluorometric method for the simultaneous detection of both intracellular and surface antigens of apoptotic peripheral lymphocytes. *Journal of Immunological Methods* 217(1-2):11-26

Lee KN, Birckbichler PJ, Fesus L (1986) Purification of human erythrocyte transglutaminase by immunoaffinity chromatography. *Preparative Biochemistry* 16(4):321-35
Lee KN, Birckbichler PJ, Patterson MK Jr. (1989) GTP hydrolysis by guinea pig liver transglutaminase. *Biochemical and Biophysical Research Communications* 162(3):1370-5
Lindroos PM, Zarnegar R, Michalopoulos GK (1991) Hepatocyte growth factor (hepatopoietin A) rapidly increases in plasma before DNA synthesis and liver regeneration stimulated by partial hepatectomy and carbon tetrachloride administration. *Hepatology* 13(4):743-50
Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997 91(4):479-89
Lorand L, Graham RM (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nature Reviews / Molecular Cell Biology* 4(2):140-56
Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362(6423):847-9
Lu S, Saydak M, Gentile V, Stein JP, Davies PJ (1995) Isolation and characterization of the human tissue transglutaminase gene promoter. *Journal of Biological Chemistry* 270(17):9748-56
Mallipeddi R (2002). Epidermolysis bullosa and cancer. *Clinical and Experimental Dermatology* 27, 616–623.
Melino G, Annicchiarico-Petruzzelli M, Piredda L, Candi E, Gentile V, Davies PJ, Piacentini M. (1994) Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells. *Molecular and Cellular Biology* 14(10):6584-96
Mevorach D, Zhou JL, Song X, Elkon KB (1998) Systemic exposure to irradiated apoptotic cells induces autoantibody production. *Journal of Experimental Medicine* 188(2):387-92
Michel MC, Kerker J, Branchek TA, Forray C (1993) Selective irreversible binding of chloroethylclonidine at alpha 1- and alpha 2-adrenoceptor subtypes. *Molecular Pharmacology* 44(6):1165-70
Mishra S, Murphy LJ (2004) Tissue transglutaminase has intrinsic kinase activity: Identification of transglutaminase 2 as an insulin-like growth factor binding protein-3 kinase. *Journal of Biological Chemistry* [Epub. ahead of print]
Molberg O, Mcadam SN, Korner R, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Noren O, Roepstorff P, Lundin KE, Sjostrom H, Sollid LM (1998) Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nature Medicine* 4(6):713-7
Monczak Y, Trudel M, Lamph WW, Miller WH Jr. (1997) Induction of apoptosis without differentiation by retinoic acid in PLB-985 cells requires the activation of both RAR and RXR. *Blood* 90(9):3345-55
Murtaugh MP, Mehta K, Johnson J, Myers M, Juliano RL, Davies PJ (1983) Induction of tissue transglutaminase in mouse peritoneal macrophages. *Journal of Biological Chemistry* 258(18):11074-81
Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signalling complex. *Cell*. 85(6):817-27.
Nakaoka H, Perez DM, Baek KJ, Das T, Husain A, Misono K, Im MJ, Graham RM (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signalling function. *Science* 264(5165):1593-6

Nanda N, Iismaa SE, Owens WA, Husain A, Mackay F, Graham RM (2001) Targeted inactivation of Gh/tissue transglutaminase II. . *Journal of Biological Chemistry*
Nemes Z, Marekov LN, Fesus L, Steinert PM (1999) A novel function for transglutaminase 1: attachment of long-chain omega-hydroxyceramides to involucrin by ester bond formation. *Proceedings of the National Academy of Sciences USA* 96(15):8402-7
Ni R, Tomita Y, Matsuda K, Ichihara A, Ishimura K, Ogasawara J, Nagata S (1994) Fas-mediated apoptosis in primary cultured mouse hepatocytes *Experimental Cell Research* 215(2):332-7
Niikura T, Hirata R, Weil SC (1997) A novel interferon-inducible gene expressed during myeloid differentiation. *Blood Cells, Molecules, and Diseases* 23(3):337-49
Nilsson JA, Zheng X, Sundqvist K, Liu Y, Atzori L, Elfwing A, Arvidson K, Grafström RC (1998) Toxicity of formaldehyde to human oral fibroblasts and epithelial cells: influences of culture conditions and role of thiol status. *Journal of Dental Research* 77:1896–1903.
Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S. (1993) Lethal effect of the anti-Fas antibody in mice. *Nature* 364(6440):806-9
Oliverio S, Amendola A, Rodolfo C, Spinedi A, Piacentini M (1999) Inhibition of "tissue" transglutaminase increases cell survival by preventing apoptosis. *Journal of Biological Chemistry* 274(48):34123-8
Orrenius S, Zhivotovsky B, Nicotera P (2003) Regulation of cell death: the calcium-apoptosis link. *Nature Reviews / Molecular Cell Biology* 4(7):552-65
O'Toole EA (2001). Extracellular matrix and keratinocyte migration. *Clinical and Experimental Dermatology* 26, 525–530
Owen-Schaub LB, Yonehara S, Crump WL 3rd, Grimm EA (1992) DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. *Cellular Immunology* 140(1):197-205
Parameswaran KN, Cheng XF, Chen EC, Velasco PT, Wilson JH, Lorand L (1997) Hydrolysis of gamma:epsilon isopeptides by cytosolic transglutaminases and by coagulation factor XIIIa. *Journal of Biological Chemistry* 272(15):10311-7
Park KC, Chung KC, Kim YS, Lee J, Joh TH, Kim SY. (2004) Transglutaminase 2 induces nitric oxide synthesis in BV-2 microglia. *Biochemical and Biophysical Research Communications* 323(3):1055-62
Peng X, Zhang Y, Zhang H, Graner S, Williams JF, Levitt ML, Lokshin A (1999) Interaction of tissue transglutaminase with nuclear transport protein importin-alpha3. *FEBS Letters* 446(1):35-9
Penninger JM, Kroemer G. (2003) Mitochondria, AIF and caspases--rivaling for cell death execution. *Nature Cell Biology* 5(2):97-9
Persikov AV, Brodsky B (2002) Unstable molecules form stable tissues. *Proceedings of the National Academy of Sciences USA* 99(3):1101-3
Peterson LL, Zettergren JG, Wuepper KD (1983) Biochemistry of transglutaminases and cross-linking in the skin. *Journal of Investigative Dermatology* 81(1 Suppl):95s-100s
Piacentini M, Farrace MG, Piredda L, Matarrese P, Ciccosanti F, Falasca L, Rodolfo C, Giammarioli AM, Verderio E, Griffin M, Malorni W (2002) Transglutaminase overexpression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress. *Journal of Neurochemistry* 81(5):1061-72

Pitman SW, Tucker A, Sasaki C (1983). Establishment and characterisation of a human squamous head and neck cancer cell line. *Proceedings of the American Association of Cancer Research* 24, 5.
Pittoni V, Valesini G (2002) The clearance of apoptotic cells: implications for autoimmunity. *Autoimmunity reviews* 1(3):154-61
Raghunath M, Hopfner B, Aeschlimann D, Luthi U, Meuli M, Altermatt S, Gobet R, Bruckner-Tuderman L, Steinmann B (1996) Cross-linking of the dermo-epidermal junction of skin regenerating from keratinocyte autografts. Anchoring fibrils are a target for tissue transglutaminase. *Journal of Clinical Investigations* 98(5):1174-84
Rahmatullah M, Boyde TR (1980) Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clinica Chimica Acta* 107(1-2):3-9
Reddien PW, Cameron S, Horvitz HR (2001) Phagocytosis promotes programmed cell death in C. elegans. *Nature* 412(6843):198-202
Ritter SJ, Davies PJ (1998) Identification of a transforming growth factor-beta1/bone morphogenetic protein 4 (TGF-beta1/BMP4) response element within the mouse tissue transglutaminase gene promoter. *Journal of Biological Chemistry* 273(21):12798-806
Rodolfo C, Mormone E, Matarrese P, Ciccosanti F, Farrace MG, Garofano E, Piredda L, Fimia GM, Malorni W, Piacentini M. (2004) "Tissue" transglutaminase is a multifunctional BH3-only protein. *Journal of Biological Chemistry* [Epub ahead of print]
Rose DM, Fadok VA, Riches DW, Clay KL, Henson PM (1995) Autocrine/paracrine involvement of platelet-activating factor and transforming growth factor-beta in the induction of phosphatidylserine recognition by murine macrophages. *Journal of Immunology* 155(12):5819-25
Rosen A, Casciola-Rosen L (1999) Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. *Cell Death and Differentiation* 6(1):6-12
Rouayrenc JF, Boise LH, Thompson CB, Privat A, Patey G (1995) Presence of the long and the short forms of Bcl-X in several human and murine tissues. *Comptes Rendus de l'Académie des Sciences - Series III* 318(5):537-40
Rust C, Gores GJ (2000) Apoptosis and liver disease. *American Journal of Medicine* 108(7):567-74.
Sanders RJ, Mainiero F, Giancotti FG (1998) The role of integrins in tumorgenesis and metastasis. *Cancer Investigation* 16, 329–344
Sarang Z, Haig Y, Hansson A, Vondracek A, Warngard L, Grafstrom RC (2003) Microarray assessment of fibronectin, collagen and integrin expression and the role of fibronectin-collagen coating in the growth of normal, SV40 T-antigen-immortalised and malignant human oral keratinocytes. *Alternatives to Laboratory Animals* 31, 575-585
Sartorius U, Schmitz I, Krammer PH. (2001) Molecular mechanisms of death-receptor-mediated apoptosis. *Chembiochem* 2(1):20-9
Savill J, Fadok V (2000) Corpse clearance defines the meaning of cell death. *Nature* 407(6805):784-8
Schmitz I, Walczak H, Krammer PH, Peter ME (1999) Differences between CD95 type I and II cells detected with the CD95 ligand. *Cell Death and Differentiation* 6(9):821-2
Shi Y (2001) A structural view of mitochondria-mediated apoptosis. *Nature Structural Biology* 8(5):394-401
Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, *et al*. (1992) Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359(6397):693-9

Simpson DW, Roth R, Loose LD (1979) A rapid, inexpensive and easily quantified assay for phagocytosis and microbicidal activity of macrophages and neutrophils. *Journal of Immunological Methods* 29(3):221-6
Song L-W, Wu J, Kojima S, Zern MA. (2001) Translocation of tissue transglutaminase from cytoplasm to nucleus plays a role in ethanol-induced apoptosis in rat hepatocytes. *Hepatology* 34(2):274A.
Sundqvist K, Kulkarni P, Hybbinette SS, Bertolero F, Liu Y, Grafstrom RC (1991) Serum-free growth and karyotype analyses of cultured normal and tumorous (SqCC/Y1) human buccal epithelial cells. *Cancer Communication* 3(10-11):331-40
Sung LA, Chien S, Fan YS, Lin CC, Lambert K, Zhu L, Lam JS, Chang LS (1992) Human erythrocyte protein 4.2: isoform expression, differential splicing, and chromosomal assignment. *Blood* 79(10):2763-70
Szegezdi E, Szondy Z, Nagy L, Nemes Z, Friis RR, Davies PJ, Fesus L. (2000) Apoptosis-linked in vivo regulation of the tissue transglutaminase gene promoter. *Cell Death and Differentiation* 7(12):1225-33
Szegezdi E, Kiss I, Simon A, Blasko B, Reichert U, Michel S, Sandor M, Fesus L, Szondy Z. (2003) Ligation of retinoic acid receptor alpha regulates negative selection of thymocytes by inhibiting both DNA binding of nur77 and synthesis of bim. *Journal of Immunology* 170(7):3577-84.
Szondy Z, Molnar P, Nemes Z, Boyiadzis M, Kedei N, Toth R, Fesus L (1997a) Differential expression of tissue transglutaminase during in vivo apoptosis of thymocytes induced via distinct signalling pathways. *FEBS Letters* 404(2-3):307-13
Szondy Z, Reichert U, Bernardon JM, Michel S, Toth R, Ancian P, Ajzner E, Fesus L (1997b) Induction of apoptosis by retinoids and retinoic acid receptor gamma-selective compounds in mouse thymocytes through a novel apoptosis pathway. *Molecular Pharmacology* 51(6):972-82
Szondy Z, Reichert U, Bernardon JM, Michel S, Toth R, Karaszi E, Fesus L (1998) Inhibition of activation-induced apoptosis of thymocytes by all-trans- and 9-cis-retinoic acid is mediated via retinoic acid receptor alpha. *Biochemical Journal* 331 (Pt 3):767-74
Takahashi N, Takahashi Y, Putnam FW. (1986) Primary structure of blood coagulation factor XIIIa (fibrinoligase, transglutaminase) from human placenta. *Proceedings of the National Academy of Sciences USA* 83(21):8019-23
Thomas GJ, Jones J, Speight PM (1997) Integrins and oral cancer. *Oral Oncology* 33(6):381-8
Thomas, GJ and Speight PM (2001) Cell adhesion molecules and oral cancer. *Critical Reviews in Oral Biology and Medicine* 12, 479–498
Vaux DL, Korsmeyer SJ (1999) Cell death in development. *Cell* 96(2):245-54
Vondracek M, Weaver DA, Sarang Z, Hedberg JJ, Willey JC, Warngard L, Grafstrom RC (2002) Transcript profiling of enzymes involved in detoxification of xenobiotics and reactive oxygen in human normal and simian virus 40 T antigen-immortalized oral keratinocytes. *International Journal of Cancer* 99(6):776-82
Wands JR, Carter EA, Bucher NL, Isselbacher KJ. (1979) Inhibition of hepatic regeneration in rats by acute and chronic ethanol intoxication. *Gastroenterology* 77(3):528-31
Wanner GA, Mica L, Wanner-Schmid E, Kolb SA, Hentze H, Trentz O, Ertel W. (1999) Inhibition of caspase activity prevents CD95-mediated hepatic microvascular perfusion failure and restores Kupffer cell clearance capacity. *FASEB Journal* 13(10):1239-48
Woronicz JD, Calnan B, Ngo V, Winoto A (1994) Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* 367(6460):277-81

Wozniak M, Fausto A, Carron CP, Meyer DM, Hruska KA (2000) Mechanically strained cells of the osteoblast lineage organize their extracellular matrix through unique sites of alphavbeta3-integrin expression. *Journal of Bone and Mineral Research* 15(9):1731-45
Wu J, Liu SL, Zhu JL, Norton PA, Nojiri S, Hoek JB, Zern MA (2000) Roles of tissue transglutaminase in ethanol-induced inhibition of hepatocyte proliferation and alpha 1-adrenergic signal transduction. *Journal of Biological Chemistry* 275(29):22213-9
Woodley DT, Chen JD, Kim JP, Sarret Y, Iwasaki T, Kim YH, O'Keefe EJ (1993) Reepithelialization. Human keratinocyte locomotion. *Dermatologic Clinics* 11, 641–646
Yin X-M, Wang Y, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 400(6747):886-91
Ziober BL, Kramer RH (1996) Identification and characterization of the cell type-specific and developmentally regulated alpha7 integrin gene promoter. *Journal of Biological Chemistry* 271(37):22915-22

List of publications

In extenso publications related to the thesis:

1. Szondy Z*, **Sarang Z***, Molnar P, Nemeth T, Piacentini M, Mastroberardino PG, Falasca L, Aeschlimann D, Kovacs J, Kiss I, Szegezdi E, Lakos G, Rajnavolgyi E, Birckbichler PJ, Melino G, Fesus L (2003) Transglutaminase $2^{-/-}$ mice reveal a phagocytosis-associated crosstalk between macrophages and apoptotic cells. *Proceedings of the National Academy of Sciences USA* 100(13):7812-7 IF: 10.7

*: these authors contributed equally to the work

2. Sarang Z, Molnar P, Nemeth T, Gomba S, Kardon T, Melino G, Cotecchia, Fesus L, Szondy Z Tissue transglutaminase (TG2) acting as G protein protects hepatocytes against Fas-mediated cell death

In revision (Hepatology). IF: 9.503

3. Sarang Z, Haig Y, Hansson A, Vondracek M, Warngard L, Grafstrom RC (2003) Microarray assessment of fibronectin, collagen and integrin expression and role of fibronectin/collagen-coating for growth of normal, SV40 T antigen-immortalized and malignant human oral keratinocytes. *Alternatives to Laboratory Animals* (31):575-585 IF: 2.022

Other in extenso publication:

1. Hedberg JJ, Grafstrom RC, Vondracek M, **Sarang Z**, Warngard L, Hoog JO (2001) Micro-array chip analysis of carbonyl-metabolising enzymes in normal, immortalised and malignant human oral keratinocytes. *Cellular and Molecular Life Sciences* 58(11):1719-26 IF: 4.539

2. Dressler D*, **Sarang Z***, Szondy Z, Engelhart K, Grafstrom RC (2002) Expression of retinoid-related genes in serum-free cultures of normal, immortalized and malignant human oral keratinocytes. *International Journal of Oncology* 20(5):897-903 IF: 2.931

*: these authors contributed equally to the work

3. Vondracek M, Weaver DA, **Sarang Z**, Hedberg JJ, Willey JC, Warngard L, Grafstrom RC (2002) Transcript profiling of enzymes involved in detoxification of xenobiotics and reactive oxygen in human normal and simian virus 40 T antigen-immortalized oral keratinocytes.

International Journal of Cancer 99(6):776-82 IF: 4.056

4. Hansson A, Bloor BK, **Sarang Z**, Haig Y, Morgan PR, Stark HJ, Fusenig NE, Ekstrand J, Grafstrom RC (2003) Analysis of proliferation, apoptosis and keratin expression in cultured normal and immortalized human buccal keratinocytes. *Europen Journal of Oral Sciences* 111(1):34-41 IF: 1.218

Conferences

Studies of toxicity, gene expression and transformation-potential of formaldehyde in cultured normal and immortalized human oral keratinocytes

Zs. Sarang, J. A. Nilsson, J. Hedberg and RC. Grafström

43rd International Meeting of the European Tissue Culture Society, Granada, Spain, 2001 (poster presentation)

Influences of retinoic acid and related derivatives on proliferation, apoptosis and differentiation of cultured human oral keratinocyte lines

Zs. Sarang, Zs. Szondy and RC. Grafström

44th International Meeting of the European Tissue Culture Society, Oxford, England, 2002 (oral presentation)

Retinoid-kapcsolt gének expressziója normál, immortalizált és malignus keratinociták szérummentes sejttenyészeteiben

Sarang Zsolt, Dirk Dressler, Roland Grafström és Szondy Zsuzsa

8th Conference of the Hungarian Biochemical Society, Tihany, Hungary, 2003 (poster presentation)

Influences of retinoic acid and related derivatives on proliferation, apoptosis and differentiation of cultured human oral keratinocyte lines

Z. Sarang, Z. Szondy and RC. Grafström

11th Euroconference on Apoptosis, Ghent, Belgium 2003 (poster presentation)

A szöveti transzglutamináz védelmet nyújt a májsejteknek a Fas receptor közvetítette sejthalállal szemben

Sarang Zsolt, Molnár Péter, Németh Tamás, Gomba Szabolcs, Kardon Tamás, Gerry Melino, Susanna Cotecchia, Fésüs László és Szondy Zsuzsa

9th Conference of the Hungarian Biochemical Society, Sopron, Hungary 2004 (poster presentation)

Tissue transglutaminase protects hepatocytes against Fas-mediated cell death

Zsolt Sarang, Péter Molnár, Tamás Németh, Szabolcs Gomba, Tamás Kardon, Gerry Melino, Susanna Cotecchia, László Fésüs and Zsuzsa Szondy

12th Euroconference on Apoptosis, Chania, Greece 2004 (poster presentation)