

Summary of Thesis for the degree of Doctor of Philosophy (Ph.D.)

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

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

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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 thesis consists of two separate sections: the first part comprises my work completed at the Department of Biochemistry and Molecular Biology; the second part contains experiments carried out at Karolinska Institute.

Part I.

Introduction

Hallmarks of apoptosis

Apoptosis is an active form of cell death characterized by a sequence of morphological events: nuclear and cytoplasmic condensation with blebbing of the plasma membrane and exposure of phosphatidylserine (PS), forming of apoptotic bodies. These are recognized and engulfed rapidly by neighbouring cells or macrophages. Apoptosis can be triggered either by cytotoxic agents or by receptor mediated signals. These signals act via different signalling pathways in mammalian cells: the extrinsic, receptor-mediated, the intrinsic, mitochondria-mediated, caspase-2-dependent and the caspase independent pathways. In the receptor-mediated pathway, ligation of death receptors is followed by the formation of the DISC, which results in the activation of pro-caspase-8. In type I cells, caspase-8 activates pro-caspase-3, which cleaves target proteins, leading to apoptosis. In type II cells, caspase-8 cleaves Bid, which, in turn, induces mitochondria-mediated pathway. This cell death pathway is controlled by Bcl-2 family proteins. During apoptosis, besides the morphological changes, the cells also expose molecules on their surface, which serve as “eat me” signal for phagocytes.

Removal of apoptotic cells

All the apoptotic bodies formed are rapidly removed by neighboring cells or macrophages. Recently a number of surface receptors have been identified that participate in the recognition and uptake of apoptotic cells. One of them is the phosphatidylserine (PS) receptor. The uptake of apoptotic cells requires re-organization of cytoskeleton, which is regulated by the activity of rac proteins. Insufficient phagocyte capacity for apoptotic cells can result 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 . 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.

Tissue transglutaminase (TGase2)

TGase2 is a member of a family of thiol- and Ca²⁺-dependent acyl transferases that establish ϵ -(γ -glutamyl)lysine cross-linkages and/or incorporate polyamines and histamine into proteins. In addition to its cross-linking function TGase2 can also hydrolyze ATP and GTP. The guanosine nucleotide binding form of TGase2, known as G_{alpha(h)}, participates in several signal transducing pathway including the α_1 -b adrenergic receptor (ADR) signalling. TGase2 was shown to sensitize cells towards apoptosis and to be both induced and activated during apoptosis in many experimental models. However, apoptosis has been shown to proceed without the induction of TGase2 expression and TGase2 can also protect against apoptosis. 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.

Regulation of TGase2 expression and activity

Even though the core TGase2 promoter is constitutively active, the intact TGase2 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 increased concentration of intracellular Ca²⁺. GTP can also modulate the cross-linking activity of TGase2 by reducing its affinity

for Ca^{2+} . 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. In negative selection, autoreactive clones bearing TCR which have a "high" affinity for self-antigen are deleted, i.e. induced to undergo apoptosis. Apoptosis can be induced by various signalling pathways in immature T cells: glucocorticoids act through glucocorticoid receptors, retinoids through $\text{RAR}\gamma$ while TCR activates the transcription factor *nur77*, ionizing radiation *p53* and FasL.

Apoptosis in the liver

Deregulation of the apoptotic program is pathophysiologically involved in many liver diseases such as disease, viral hepatitis, hepatocellular carcinoma, alcoholic and cholestatic liver diseases. Fas induced apoptosis proposed as a determining mechanism in all these diseases. Hepatocytes belong to type II cells in which apoptosis triggered by FasL or agonistic anti-Fas antibodies goes through the mitochondria. cFLIP, a catalytically inactive caspase-8/-10 homologue, can interrupt the Fas-triggered apoptotic pathway and block the Fas-induced cell death. 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. The major organ affected is the liver and the massive hepatocyte apoptosis is responsible for the animal death. 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 cFLIP. Besides hepatocytes, hepatic sinusoidal endothelial cells are also susceptible to Fas-mediated apoptosis further contributing to the Fas-induced hepatic failure.

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.

Aim of the study

The overall aim 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 and their wild-type counterparts were obtained from Gerry Melino's laboratory. α_1 -b-adrenoceptor KO (ADR^{-/-}) 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 anti-CD3 mAb, DXM, exposed to γ -irradiation or Jo2 anti-CD95 mAb. In some experiments neutralizing anti-TGF- β mAb was injected prior to the treatment and a second dose at the time of apoptosis induction. 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 stained either with PE labelled anti-CD4 and Cy5-conjugated anti-CD8 antibodies or with FITC-labelled annexin-V. Cell-bound fluorescence was analyzed by flow cytometer.

Thymocyte apoptosis in vitro. Thymocyte suspensions were prepared by mincing thymus of four weeks old mice. After washing thymocytes were diluted to a concentration of 10⁶ cells/ml and cultured at 37°C / 5% CO₂. Apoptosis was induced by addition of DXM, anti-CD3 mAb, etoposide or anti-CD95 mAb. In some experiments recombinant TGF- β 1 was also added. After 6 hrs, the extent of cell death was determined by 7-aminoactinomycin D uptake.

Induction of hepatocyte apoptosis in vivo was carried out at Mauro Piacentini's laboratory, University of Tor Vergata, Rome. Hepatocyte apoptosis was induced by intravenous injection of PbNO₃. 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 (HM). The percentage of apoptotic cells was assessed by light microscopy.

Determination of TGase activity. Thymus was homogenized and TGase activity was measured by detecting the incorporation of [³H]putrescine into N,N'-dimethylcasein. Results are given as nmoles of [³H]putrescine incorporated per mg protein and per hour.

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. Electrophoresis was performed in a 10% SDS-polyacrylamide gel. Separated proteins were electroblotted and probed with monoclonal anti-TGase2 (CUB7402), polyclonal anti-TGase2 antibodies to TGase2. Bound antibodies were visualized using ECL detection system.

Determination of the expression of TGase1,2,3,5,7. Expression of TGase1,2,3,5,7 was determined by real-time quantitative PCR carried out on an ABI PRISM 7700 Sequence Detection System using the 5' nuclease assay at Daniel Aeschlimann's laboratory in Wales. Ribosomal protein S26 was used as an internal reference of housekeeping gene transcription for normalization between different cDNA samples.

Transmission electronmicroscopy was carried out at the Department of Pathology. For transmission electronmicroscopy analysis thymus samples and livers were fixed, cut in small pieces and postfixated with OsO₄. Tissue samples were then dehydrated and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead nitrate and observed under electronmicroscope.

Macrophage phagocytosis assays. Macrophages were harvested from adult mice by peritoneal lavage and seeded on 2% gelatine treated 24-well plates. 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. For generation of apoptotic cells, thymocytes were induced to die by addition of calcium ionophore for 6 hrs. Target cells were placed on top of the macrophages at a concentration of 10⁷ cells/ml. After 1 h of co-culture thymocytes that had

not been taken up were washed away, while the adherent cells were fixed. After rinsing cells were „stained” with osmium tetroxide and studied by phase contrast microscopy. For visualising *Listeria monocytogenes* uptake, bacteria were fluorescein-labelled with CellTracker green before addition to macrophage cultures. Cells were fixed with paraformaldehyde and analyzed using epifluorescence microscopy. Uptake of *Saccharomyces cerevisiae* was measured by counting, microscopically, the number of methylene blue positive yeast within the macrophages.

Detection of rheumatoid factor type autoantibodies was carried out at the Department of Immunology. Autoantibodies specific for mouse IgG isotypes were detected by solid phase ELISA.

Detection of IgM-containing immune complexes. Kidney was removed, snap-frozen with isopentane in liquid nitrogen. Cryosections were cut, fixed in cold acetone, and incubated with FITC-labelled anti-mouse IgM. After rinsing in PBS, slides were mounted in buffered glycerol.

Determination of serum urea concentration. Samples were mixed with color reagent in 1:3 ratio and incubated at 85 °C for 30 minutes followed by incubation at 12 °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 anti-Fas antibody. In some experiments TGase2^{+/+} mice were treated for a week with a daily dose of chloroethylclonidine (CEC), an adrenoceptor antagonist, to detect changes in the Bcl-x_L expression and in the *in vitro* Fas sensitivity of hepatocytes.

Hepatocyte culture experiments. Hepatocytes were isolated at the Department of Medical Chemistry, SOTE. Isolated hepatocytes were cultured at 37°C/5% CO₂ 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⁺ and propidium iodide negative cells.

Determination of cell surface expression of Fas on hepatocytes of WT and TGase2^{-/-} mice. Freshly isolated hepatocytes were washed in PBS and stained with a PE-labelled anti-Fas antibody. Fluorescence was analyzed on flow cytometer.

Determination of Bcl-x_L and cFLIP(L) expression in the liver of wild-types, TGase2^{-/-} and ADR^{-/-} mice. Samples from liver tissue homogenates containing 1 mg/ml protein were mixed with an equal volume of sample buffer. Electrophoresis was performed in case of Bcl-x_L detection in a 12% and in case of FLIP detection in 10% SDS-polyacrylamide gel. Separated proteins were electroblotted and probed with anti-Bcl-x_L and anti-FLIP antibodies. Bands were visualized by the ECL technique.

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 HM. For transmission electronmicroscopy (TEM) analysis livers were fixed with a modified Karnovsky fixative, followed by osmication. Tissue samples were then dehydrated and embedded in Araldit resin. Ultrathin sections were “stained” with uranyl acetate and lead nitrate and observed under a JEOL 1010 transmission electronmicroscope.

TUNEL staining was carried out at the Department of Dermatology and Venerology. Livers from anti-Fas antibody-treated mice were removed, washed with physiological saline, formalin fixed, embedded in paraffin and stained with the APOPTAG *in situ* apoptosis detection kit.

Determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Sera were collected following anti-Fas antibody injection and 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, also induced the *in vivo* expression of TGase2 mRNA with a concomitant increase in the enzyme protein and activity in the WT animals. The truncated TGase2 mRNA was expressed in TGase2^{-/-} mice, but the respective protein was not detectable. From the fact that increase in TGase2 mRNA level after apoptosis induction was lower in the TGase2^{-/-} mice than in the WT ones we concluded that its transcriptional activation is TGase2-dependent. Similar results were obtained with other TGases. We also found that after apoptosis induction in TGase2^{-/-} mice the loss in thymus weight and cellularity was reduced as compared to WT thymus. Measuring the changes in the percentage of the annexin V⁺ (free apoptotic) thymocytes after apoptosis induction and histological examination of thymus section revealed that the reduced loss of thymic cells in the KO mice was due a reduced rate of phagocytosis and prolonged persistence of apoptotic cells.

Defective clearance of apoptotic cells is accompanied with inflammatory reaction in the liver of TGase2^{-/-} mice.

Histological examination of livers from PbNO₃, known to induce hepatocyte proliferation and subsequent apoptosis, treated animals proved that the reduced phagocytosis in TGase2^{-/-} mice was not restricted to the thymus. 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 in the TGase2^{-/-} but not in the WT livers.

The production of active TGF- β 1 promotes phagocytosis of apoptotic cells by macrophages and requires TGase2.

Using peritoneal macrophages and thymocytes from WT and TGase2^{-/-} animals we demonstrated that TGase2 is required for proper phagocytosis in the macrophages. Additionally, the

deficiency in phagocytosis seems to be restricted to phagocytosis of apoptotic cells as TGase2^{-/-} macrophages showed a normal ability to ingest bacteria, yeast or opsonized non-apoptotic thymocytes.

Macrophages are known to express TGase2, which is required for the activation of latent TGF-β1. Using cell culture medium transfer experiments we demonstrated that TGF-β produced by WT macrophages can enhance phagocytotic capacity of TGase2^{-/-} macrophages.

TGF-β1 is responsible for the induction of TGase2 expression during in vivo apoptosis. To test the hypothesis that TGF-β is responsible for the upregulation of TGase2 *in vivo* we injected WT mice with neutralizing anti-TGF-β1,2,3 antibodies. In the presence of anti-TGF-β antibodies the induction of the TGase2 activity after anti-CD3, DXM treatment or γ-irradiation was significantly reduced.

TGF-β1 promotes apoptosis of thymocytes. Addition of TGF-β1 promoted *in vitro* death of WT thymocytes induced by DXM, anti-CD3 mAb and etoposide. This effect of TGF-β1 was not related to the up-regulation of TGase2 in apoptotic cells, because it promoted thymocyte apoptosis in TGase2^{-/-} mice to a similar degree.

TGase2^{-/-} mice develop autoantibodies, splenomegaly and immune complex glomerulonephritis. We then investigated the long-term effects of the TGase2 deficiency. We found that one year old TGase2^{-/-} mice had autoantibodies against nuclear components and smooth muscle cells, while none of the age matched control WT mice were tested positive. Moreover, TGase2^{-/-} mice with autoantibodies developed splenomegaly as well. At the age of 15 months, KO mice demonstrated antinuclear antibodies and anti-cytoskeletal positivity, and some of the animals were terminally ill with as immune complex glomerulonephritis. Glomerular dysfunction was indicated by high serum concentrations of urea. At the same age, none of the WT animals had signs of kidney damage.

TGase2^{-/-} animals are more sensitive to Fas-induced killing than WT animals

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. Following intraperitoneal anti-Fas injection, the size of the CD4⁺CD8⁺ thymocyte pool decreased in WT and TGase2^{-/-} 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. 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 survive the otherwise for the WT mice sub-lethal dose of anti-Fas. We decided to characterize this phenomenon.

Wild-type hepatocytes die primarily by necrosis, while TGase2^{-/-} cells die by apoptosis following Fas engagement in vivo by a non-lethal dose of anti-Fas antibodies. Haematoxyline-eosine staining and transmission electronmicroscopical examination of liver sections from mice treated with anti-Fas antibody revealed that in the liver of WT animals hepatocytes died primarily by necrosis while in liver of TGase2^{-/-} mice there were signs of heavy hepatocyte apoptosis accompanied with massive hemorrhagic suffusions, suggesting also the development of disseminated endothelial cell damage. In support of this there were numerous TUNEL positive hepatocytes in the liver of TGase2^{-/-} mice while only endothelial cells were stained positive in WT livers. Additionally, 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.

The increased damage of TGase2^{-/-} hepatocytes following anti-Fas treatment *in vivo* was further confirmed by the significantly elevated serum AST and ALT levels in KO mice as compared to WT mice.

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 WT and KO 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.

TGase2^{-/-} hepatocytes demonstrated both an increased rate of spontaneous death in culture and an increased sensitivity to Fas-mediated death, as compared to WT 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 WT and TGase2^{-/-} hepatocytes.

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 expression in hepatocytes were also determined. Bcl-x_L expression, but not cFLIP(L) expression, was found to be decreased in TGase2^{-/-} hepatocytes, as compared to the WT cells.

Inhibition of the α_1 -b-adrenerg receptor leads to both down-regulation of the Bcl-x_L expression and increased Fas-sensitivity of hepatocytes. *In vivo* pretreatment of WT mice with chloroethylclonidine (CEC), an ADR antagonist, decreased the Bcl-x_L expression in hepatocytes and rendered them *in vitro* sensitive to anti-Fas similarly to TGase2^{-/-} hepatocytes. 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.

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 ($ADR^{-/-}$) was also determined. The WT partners for the $ADR^{-/-}$ 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. Consequently, these mice are more susceptible to anti-Fas antibody and therefore the dosage of anti-Fas had to be lowered.

ADR^{-/-} mice are also more susceptible to Fas-induced killing than their wild-type counterparts. While 80% the WT animals survived the anti-Fas antibody, only 10% of the knock-outs survived for 48 hrs following administration of the same dose of anti-Fas antibodies.

Livers from ADR^{-/-} mice show considerably more damage than WT livers after anti-Fas engagement. Livers were examined in case of the $ADR^{-/-}$ mouse after the time of death or in case of WT mouse at the time point when the $ADR^{-/-}$ mouse died. Both the WT and the $ADR^{-/-}$ liver showed histological evidence of parenchymal degradation and hepatocyte apoptosis but this was more pronounced in the $ADR^{-/-}$ liver. Increased damage of $ADR^{-/-}$ livers as compared to their wild-types was also demonstrated by the higher number of TUNEL positive liver cells on tissue sections and by the higher serum ALT and AST levels.

In accordance with our previous finding that CEC-treatment downregulated Bcl-x_L levels, ADR deficient livers also expressed lower levels of Bcl-x_L than their WT counterparts. Interestingly $ADR^{+/+}$ mice also expressed lower levels of cFLIP(L) and Bcl-x_L than the TGase2^{+/+} mice, which might explain their increased Fas sensitivity. On the other hand no difference was found in the cFLIP(L) expressions of the $ADR^{+/+}$ or $ADR^{-/-}$ hepatocytes.

Discussion

In this study, we investigated both the *in vitro* and *in vivo* apoptosis program in the absence of TGase2. Our finding, in agreement with previous results, was that loss of TGase2 did not affect the *in vitro* apoptosis of thymocytes. However, after *in vivo* apoptosis induction, where TGase2 is normally upregulated, a reduced cell death was detected in the thymi of TGase2^{-/-} mice. This might be the result of the fact that in the absence of TGase2 thymocytes activate their apoptosis program slower. TGase2 was shown to sensitize cells towards apoptosis through the mitochondrial pathway. Secondly, we have found that TGF- β promotes death of thymocytes in cultures. 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 absence of TGase2 TGF- β activation is impaired and this might lead to a slower apoptosis rate.

Alteration of the rate of cell death was not restricted to the thymus. Treatment of WT and TGase2^{-/-} mice with anti-Fas revealed another consequence of TGase2 deficiency. The liver of mice was found to be particularly sensitive *in vivo* to the anti-Fas antibody, which caused massive liver apoptosis. 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 primarily 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 later hepatocytes showed necrotic morphology probably because of the oxygen and nutrition depletion due to the endothelial cell damage and microcirculation failure. 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, 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 and its overexpression was reported to be protective against Fas induced cell death we have investigated Bcl-x_L expression and found that TGase2^{-/-} hepatocytes expressed less Bcl-x_L than WT cells.

TGase2 as G_α(h) protein was shown to mediate ADR signalling in the liver. Adrenergic agonists modulate not only the metabolism but can act as mitogens in hepatocytes. Moreover α₁-adrenergic receptors were reported to be involved in proliferation of hepatocytes 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 was shown to 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. WT 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 α₁-b adrenergic signalling in the regulation of hepatocyte cell death.

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 free apoptotic cells in the thymus of TGase2^{-/-} mice suggesting that the phagocytosis of apoptotic cells is defective in these mice. The defect in clearance of apoptotic cells was also evident after PbNO₃ induced liver hyperplasia suggesting that it is not an organ-specific defect. Reduced phagocytosis was restricted only to apoptotic cells. 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.

Recent studies suggest that an intensive cross-talk between phagocytes and apoptotic cells ensures proper apoptosis and efficient uptake of apoptotic cells 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, TGF- β or nitric oxide. 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 and S19 ribosomal protein released from apoptotic cells were shown as an effective chemotactic factor for monocytes. Furthermore, upon binding to phagocytes, apoptotic corpses re-program them to shut down pro-inflammatory cytokine production. TGase2 participates on both sides in this cross-talk. Activation of TGF- β , synthesis of NO and formation of the cross-linked S19 ribosomal protein were all shown to depend on TGase2 enzyme activity. Besides these roles, TGase2 also seems to participate in the exposure of PS, as our 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.

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 PbNO₃ treatment. The enhanced inflammatory response in TGase2^{-/-} liver can be partly explained by the reduction in the anti-inflammatory TGF-β activity. As a consequence of impaired phagocytosis, free apoptotic corpses accumulated in the thymus and liver of TGase2^{-/-} mice. Moreover, apoptotic cells express certain nuclear antigens their surface, 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 results in the transient production of autoantibodies, and defects in clearance of dead cells are associated with autoimmunity. Indeed, the long-term consequence of the defect in TGase2 function was the development of autoimmunity characterized by the appearance of autoantibodies, increased titers of IgG type anti-IgG2a anti-bodies and increased number of CD4⁺ and B cells. This was accompanied by splenomegaly and glomerulonephritis. 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.

Taken together, our data suggest that TGase2 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 proinflammatory 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 α_1 -b-adrenoceptor deficient mice proved that TGase2 as G_{alpha(h)} protein in α_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.

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 mainly by fibroblasts 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. 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. They are major structural proteins in the extracellular matrix, making up about one-third of protein mass in higher animals. These basic structures organize the extracellular matrix and give it resilience. Fibronectin, the first well-characterized adhesive protein, is a dimer 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. 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. Until now 18 alpha and 8 beta subunits have been identified. From these subunits, at least 25 distinct integrins are formed.

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. 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). Development of oral and other cancers is characterized by the alteration of specific genes, such as p53, p27, p16, and cyclin D-1. 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. 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. 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. Furthermore, SVpgC2a showed elevated proliferation and apoptosis similarly to oral preneoplastic lesions. 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. Normal oral keratinocytes were isolated from clinically healthy human buccal tissue (used with the approval of the Karolinska Institute ethical committee) by trypsin digestion. Cells were resuspended in serum-free growth medium (EMHA) and plated onto fibronectin-collagen (FN-COL)-coated dishes. The immortal cell line SVpgC2a, derived by transfection and stable integration of the SV40 T antigen into buccal keratinocytes, and the buccal carcinoma cell line SqCC/Y1 were also cultured EMHA.

Preparation of labelled cRNA. Total RNA was prepared with RNeasy from each type of cells. Double-stranded cDNA was synthesized and labelled cRNA was *in vitro* transcribed from the cDNA as template incorporating biotinylated CTP and UTP. The cRNA was purified with RNeasy affinity columns.

Array hybridization. To determine gene expressions, microarray chips with oligonucleotides corresponding to over 12000 human transcripts were hybridized with labelled cRNA from NOK, SVpgC2a and SqCC/Y1 cultures, respectively. Following hybridization the arrays were washed, stained with streptavidin-phycoerythrin, washed again and subsequently scanned at 570nm using a HP gene array scanner. Microarray data were analyzed with GeneChip®4.0 software, then imported, sorted and visualized in Microsoft Excel, Cluster and TreeView softwares, respectively.

Cell Growth Assessment in Mass Culture. Net growth, as indicated by the total cell number, was determined under a Nikon TMS inverted microscope. At the indicated time points, the mean number of cells per mm² was calculated in 10 randomly selected fields.

Colony-Forming Efficiency (CFE). The cells were seeded and incubated for another 8 days in EMHA. The cultures were then fixed in formalin and stained with crystal violet. The mean CFE was determined from duplicate

dishes and based on microscopic counting of the colonies. Individual cell clones were considered a colony when containing at least 12 cells.

Results

Oligonucleotide microarray. Fibronectin transcripts were abundant in all cell lines, whereas the levels of collagen chains and integrin subunits varied. We found an increased expression of collagens and decreased expression of integrins, in the immortalised SVpgC2a cells, while the malignant SqCC/Y1 resembled rather the NOK cells in regard of the expression of these molecules. 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.

Effect of fibronectin-collagen coating on cell growth. 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.

Effect of fibronectin-collagen coating on cell CFE. 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.

Effect of fibronectin-collagen coating on cell migration. The colonies from all the cell lines were reproducibly more dispersed on FN-COL-coated surfaces, with a larger surface area per colony. This effect of FN-COL was consistently seen in all the NOK, SVpgC2a and SqCC/Y1 cultures tested.

Discussion

We used the oral keratinocyte lines NOK, SVpgC2a and SqCC/Y1 under serum-free culture conditions to investigate the requirement for, and expression of, ECM proteins (fibronectin and collagen), and ECM protein receptors (integrins) for 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. Oral SCC development may involve significant changes in the expression of integrins and ECM proteins. The expression of collagen chains in SqCC/Y1 cells, which were derived from a well-differentiated buccal SCC, was similar to that in NOKs, although decreased levels of types COL7A1 and COL17A1 and increased level of COL6A1 were observed. Notably, SCC may arise as a complication of inherited epidermolysis bullosa, a disease that is characterised by the functional loss of these collagens. Loss of COL7A1 is also common in highly differentiated SCCs and may serve as a marker for invasiveness. In line with this, SqCC/Y1 cells can also grow invasively in organotypic culture. 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. Integrin expression is commonly altered in oral SCC, although primarily in poorly differentiated lesions. 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 with 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. These alterations may contribute to the poorly differentiated, basaloid phenotype of SVpgC2a cells, and also to the low coherence among cells in regenerated

epithelia and the loose attachment of monolayers to tissue culture plastic. The detection of abundant fibronectin transcripts in all the cell lines tested agreed with previous studies on normal and transformed epidermal keratinocyte lines.

Keratinocyte proliferation is often coupled with migration, but can occur independently. Previous work has shown that FN–COL coating can increase the primary culture yield of NOKs, involving increased CFE, growth rates and migration. 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 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. 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. 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.

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. 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. 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.

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

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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 szérumentes sejtenyészeteiben

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)