EXPERIMENTAL STUDIES ON THE RELATIONSHIPS BETWEEN CELL DIFFERENTIATION AND AGING USING IN VITRO CELL LINES

Thesis of university Ph. D. dissertation

by

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1. Introduction

A. A brief survey of the aging theories

The aging process was defined at phenomenological level by Strehler (1959) as a progressive, destructive, intrinsic and universal biological phenome-non. The causes of aging, as well as the possibilities of its slowing down have always been in the interest of scientists. During the recent decades, a great number of theories were born, however, only a few of them has obtained experimental support. Theories dealing with the causes and mechanisms of aging are so strongly divergent that even a very basic systemic survey of them encounters tremendous difficulties, therefore, it is not realizable without contradictions. Yet, because the construction of fundaments of such a dissertation requires a sort of survey, two attempts of the systemic summary of theories will be presented below. One of them assumes two types of theories, namely,

(1) theories explaining the aging process by the accumulation of so-called stochastic, passive errors, and
(2) theories assuming the existence of genetic aging programs, i.e., considering this process as a result of an active regulation.

A.1. Aging theories assuming passive processes

The main types of such theories will be listed below, emphasizing, how-ever, that all of them developed under the influence of each other during the last 50-60 years, and they approached often the same phenomena, but gave different importance to them.

1.1. Cross-linking theories

The main content of them is that two or more macromolecules (nucleic acids, proteins) become more and more linked to each other by the so-called cross-links, resulting in a destructive effect on the involved molecules. This concept has already more than 60 years, and is supported by numerous data. As cross-linking agents, we can list: acetaldehyde, α-ketoglutaric acid, citric acid, malic acid, fumaric acid, succinic acid, inorganic cations (various forms of Pb, Al, Cu, Fe, Mn and Zn), unsaturated fatty acids, all sorts of metabolites possessing unsaturated bonds (Björksten, 1968), as well as the oxygen free radicals (Zs.-Nagy és Floyd, 1984)

1.2. Free radical theory of aging

One of the still living “great olds” of gerontology, Denham Harman (1956) suggested that active radicals derive as harmful byproducts from the molecular oxygen, and these are of damaging effects on the cells, i.e., the elimination of these radicals may lead to a prolongation of the life. This concept, in spite of a series of necessary modifications, proved to be until now the most widely spread and useful aging theory.

1.3. Diffusion theory

Carpenter (1965) supposed that the cross-linked macromolecules act as far-reaching factors in the aging phenomena. In other words, this is a version of the cross-linking theories.

1.4. ”Collagen theory”

Verzár (1955, 1956) conducted basic experiments on the thermal denaturation of collagen. He established that part of the the H-bonds (about 10 bonds/molecule) stabilizing the young collagen molecule are transformed into covalent cross links with advancing age. Therefore, the old collagen becomes of greater mechanical and thermal stability, and contracts with greater strength, when denatured. Verzár has extended immediately these findings also to the chromatin, and established that the thermal stability of chromatin also increases, and this phenomenon takes place on the proteins, while the DNA itself remains unchanged during aging in this respect.

1.5. Error-catastrophe theory

According to Orgel (1963), the cause of aging is mainly the decrease of the fidelity of protein synthesis, i.e., erroneous proteins become synthesized with advancing age, the imperfect molecules accumulate also in the transcription and translation processes, and the accumulation of these errors may lead to a catastrophe. Although later Orgel (1970, 1973) itself has revoked his own theory, there exist followers of this idea until today.

1.6. Redundancy theory

According to Medvedev (1972), the highly repetitive DNA-sequences which are in the state of long-term repression, play a role of a sort of reserve information, and the longevity of the single species depends on the number of these repetitive sequences. This logic is essentially similar to the error-catastrophe concept, and did not result in any important understanding.

1.7. Mutation theories

These theories attribute aging to accumulated genetic defects in the cells, and represent, therefore, an alternative version of the error catastrophe theory.

1.8. Immune theories

According to Walford (1969), aging is a process in which the reticulo-endothelial system (RES) becomes more and more intolerant against the own proteins of the same organism. During the recent years, this concept has been transformed by asking, how the actual immune state may be a biomarker of the biological age. In other words, the question was asked whether one can predict the expected survival in the elderly on the basis of immune parameters. The answer is that we cannot do so on the basis of a single parameter, however, a synchronous existence of multiple alterations may indicate the outcome.

1.9. Metabolic rate theories

Pearl (1928), Mc Cay et al. (1935) and Berg (1976) concluded that the higher is the metabolic rate of an organism, the faster is its aging process. Theoretical considerations show that due to the close correlation between the metabolic rate and the oxygen consumption, this concept is linked essentially to the free radical theory of aging.

1.10. Stress-theory

The concept of stress became known on the basis of the works of J. Selye (1956). He assumed that various stress situations leave some traces in the human body, and the accumulation of them leads to aging. This concept together with the metabolic rate theories are common in claiming that the older is the organism the lower is its defense activity against the environmental damaging factors.

A.2. Theories assuming an active regulation in aging

It is a common assumption of these theories that the aging process is regulated through the DNA molecules, i.e., the gene activities are programmed in time.

2.1. Genetic program theory

Hayflick (1965, 1970, 1973, 1975) made the “observation” that human fibroblasts, if taken from the embryo, are able to perform 50 doublings in culture, then get old and dye. At the same time, fibroblasts taken from adult or elderly subjects, the doubling umber decreases to about 20. He suggested that all this reflects the existence of an aging program. After numerous debates, the conclusion was reached that the “Hayflick limit” cannot be accepted as a basis of a credible aging theory.

In spite of this situation, there exist the so called theory of replicative senescence (Smith and Pereira-Smith, 1996), according to which the normal cells possess a limited proliferative capacity, after a determined number of cell division they leave the cell cycling and become terminally differentiated, non-dividing cells. This replicative senescence is assumed to be irreversible, and although the cells do not lose their viability, one cannot stimulate them to divide any more. According to some authors, the cellular aging is a normal phe-nomenon of tumor-suppressor character, and the loss of this mechanism leads to tumor development.

2.2. Neuroendocrine theories of aging

Reiter (1994) suggests that the decreased adaptivity of the elderly, accompanied by disturbances in the biorhythms, are related to the disturbed diurnal rhythm of the melatonin level. The presence of melatonin at particular sites and receptors of the central nervous system is able to slow down the age-dependent troubles of the biorhythm regulations. It should also be noted, however, that other authors assume that all these phenomena are consequences of aging rather than causes.

2.3. Telomere-clock theory

It has been recognized that the two ends of DNA molecules have an extended region of special composition (telomeres): one can find here species-specific, numerous, tandem-fashioned, repeated short base-sequences, assuring as ort of “protection” of the chromosomal ends. Some authors claim that the telomers are shortened at each cell doubling, while others deny this (Rubin, 2002). The telomere-clock theory assumes that the cells are measuring their age (i.e., their doubling numbers) by the length of the telomeres. In other words, numerous scientists follow also here the Hayflick model. This field is actually searched very intensely, and numerous speculations are accompanied by intense debates.

A. 3. The fundaments of the theoretical gerontology

Esposito (1983) published this type of theoretical bases. He attempted first to create a system of the existing theories of aging, and classified them in 3 groups: (1) causal theories, (2) systemic theories, and (3) evolutional theories. In addition, he formulated also the basic questions of the theoretical geron-tology, which should be answered by the experimental approaches. The membrane hypothesis of aging (MHA) (Zs.-Nagy, 1978, 1994, 1997) was developed on the basis of this model, and the possible answers to the questions of theoretical gerontology were also given.

A.4. A brief outline of the MHA

Starting point of the MHA is that the continuous replacement of all the components of the living organisms is necessary, because they are continuously damaged. Therefore, one has to identify the factors (1) which compromise the integrity of molecular components all the time, (2) which eliminate continuously the damaged components, and (3) which replace the damaged and eliminated components by new ones through a de novo synthesis.

The MHA accepts that the most important damaging factors are among the oxygen free radicals, i.e., it applies the free radical theory of aging (Harman, 1956, 1981, 2001). Since, however, the polymerizing, cross-linking effects of these radicals are largely dependent on the local density of the structure, the MHA attributes a higher probability of free radical-induced damages on the more compact cell organelles, like the membranes, as compared to the free cytosol. In

addition, the cell plasma membrane is continuously exposed also to the “residual-heat”-induced damage, which is the consequence of the frequently repeated discharge of the resting potential.

The quick damage of the plasma membrane components results in a continuous decreasing tendency of the passive K^+-permeability of the cell membrane. This leads directly to an increase of the intracellular K^+-content. On the one hand, this represents an advantage, since the excitability of the membrane can be maintained in this way. On the other hand, the increased intracellular ionic strength increases the condensation (or aggregation) of the cell colloids, and also the probability of formation of intermolecular cross-links. As a consequence of all this, the cell colloids will form more and more large, aggregated particles, leading to a drop of the intracellular colloid osmotic pressure being the most important force keeping the water inside the cells. This means that the cells will lose water. This causes an increase of the relative dry-mass content throughout the life.

This process is not a particular event of aging, but it starts already during the embryonic life, and it is absolutely necessary during the ontogenesis for the growth and maturation (like the formation of the muscles, bones, etc.). Since this is an implicit, automatic process in each living cell, the dehydration trend cannot stop when reaching the optimum maturation level, but goes further even at a higher speed. When it reaches certain levels, it causes functional losses, which is already part of the aging process. The functional losses are explained by the enzyme kinetic models, according to which the increased density of the molecular environment causes a decline in the enzyme performances (Damjanovich és Somogyi, 1973; Somogyi és Damjanovich, 1975; Damjanovich et al. 1989). This involves obviously not only the metabolic, catabolic and other enzymes, but also all those performing the transcription and translation.

From the basic concept of the MHA derives that the function of the oxygen free radicals is an important factor not only in the aging organisms, but also from the beginning of the ontogenesis, during the whole life span. Part of this theoretical approach, namely the assumption that these radicals can be considered as causal factors in the start of cell differentiation, formed the basis of the present dissertation.

B. Properties of the oxygen free radicals

Definition and reactions of the free radicals

The free radicals are chemical moieties (molecules or molecule fragments) which possess an unpaired electron (unpaired spin) (Pryor, 1976). While the ionic nature of a chemical entity is determined by the relationship between the numbers of electrons and protons, free radicals, independently from the number of protons, are all the entities, which bear an unpaired spin electron on the external electron shell. Due to the presence of the unpaired spin electron, the free radicals are of paramagnetic character, and because of the strong affinity of the unpaired spin electron to pick up an electron of the opposite spin, the free radicals are strongly reactive and, correspondingly, display a short life time (Pryor, 1976).

Among the free radicals occurring in biological systems, an important place is occupied by the oxygen free radicals, termed also as ”reactive oxygen species” (ROS). For example, the superoxide ($O_2^{-\cdot}$) free radicals can exists only for a short time, since they are able to give spontaneously their superflue electron to each other, i.e., they reduce each other further reciprocally. The end product of this reaction is a divalent oxygen molecule, binding immediately two protons, giving rise to H_2O_2. The formed H_2O_2 is either split by catalase, or undergoes a Fenton reaction. Essential point of the latter is that transition metals are able to donate a further electron to it, as a consequence of which the H_2O_2 molecule will heterolyze, i.e., will split in a hydroxyl-ion (OH^-) and a hydroxy free radical ($OH\cdot$).

This latter reaction has been named after Fenton (1894), who discovered that the mixture of ferrous iron and H_2O_2 was the ever known strongest oxidative agent against organic molecules. This property is due to the ability of the $OH\cdot$ free radical to rape an electron from any adjacent moiety within about two molecular collisions.

During the early eighties started the recognition that the $OH\cdot$ free radicals deriving from the Fenton reaction are able to exert very significant influences both on proteins, amino acids and nucleic acids, etc. These trends led to the formation of a new concept regarding the true role of the oxygen free radicals, in which scientists of our Chair played an important role, and the experimental material of the present dissertation is also a part of this new concept.

<u>The non-orthodox concept of the role of oxygen free radicals</u>

Denham Harman suggested first that the oxygen free radicals are harmful byproducts of the oxidative metabolism, and the possibly complete elimination of them will prolong the survival time of all species. For sake of simplicity, this concept is called nowadays the orthodox concept.

As against to this, the non-orthodox concept states that the continuous flux of ROS is a necessary prerequisite for the maintenance of the living state, while some harmful side-effects of the same radicals cause macromolecular damages, forcing the cells to replace them from time to time.

This non-orthodox consideration of the oxygen free radicals dictates implicitly that the cell differentiation and aging are based on the same phenomena. Without such processes neither the differentiation nor the maturation of cells can take place, and furthermore, an “overdifferentiation“ above a certain level becomes a damaging process leading to aging.

Our department has studied first the effect of chemically generated OH· free radicals (through Fenton reaction) on cells cultured from the limb-bud of chicken embryo, then on Hl-60 and K562 cell lines. My experimental work was essentially a continuation of this type of experiments.

2. Aims and scope

The experiments were aimed at finding further evidences regarding the differentiation-inducer effects of the $OH^{\bullet}$ free radicals, particularly using the following approaches:

1) Introduction of a highly reproducible, sensitive method for the revelation of hemoglobin production of K562 erythroleukemia cells which could substitute the previously applied benzidin reaction (which is of carcinogenic effect).
2) Also on K562 cells, we wanted to prove the alterations in size and granulation (compactness) of cells under the effects of oxygen free radicals by an exact flow-cytometric method.
3) We intended to establish human primary fibroblast cultures from the retrobulbar muscle and fat tissue, as well as from skin.
4) We intended to measure the changes of SOD and catalase activities in normal and EOP-derived fibroblasts under the effect of Fenton reaction.

5) We demonstrated histochemically and measured spectrophometrically the changes of GAG-synthesis in the same fibroblasts under the Fenton-.treatment.
6) The cell proliferation activity was observed by immunohistochemistry in EOP-derived and control fibroblasts under the Fenton-treatment.
7) We analyzed the fibroblast-preadipocyte-adipocyte transition, and the effect of free radicals on this differentiation process on primary fibroblast cultures, as well as 3T3 and L929 cell lines, by following the fat accumulation of those cells.

3. Methods

Cell lines

K562 cell line

This is a human erythroleukemia cell line, consisting of blast type cells, being of multipotential, hematopoetic, malignant character. They may differentiate into identifiable stem-cells of the erythrocyte, granulocyte and monocyte series (Lozzio et al., 1981). They can be grown in suspension cultures, in RPMI culture medium, with 10% FCS.

L-929 cell line

This cell line derives from the connective tissue of mouse (Earle, 1940). In the history of cell culturing, the L line was the first one grown in continuous culture, and the clone 929 was the first identified cell line. The cells are of fibroblast type, grow in attached cultures, in RPMI medium, with 10% FCS.

NIH/3T3 cell line

This cell line was created from NIH Swiss mouse embryos. The cells possess a very high ability for contact inhibition. They are of fibroblast type, grow in attached culture, in DMEM medium, with 10% FCS.

Separation and culturing of a primary cell line

Primary cell lines were obtained from retrobulbar muscle and fat tissues removed during correction or decompression interventions of EOP-patients. As controls, we applied also ocular muscle biopsies obtained from strabismus operations, as well as fat tissue removed during various operations of non-EOP patients. Further control cells were obtained from human skin pieces removed from the flexor surface of the lower arm or from the abdominal area during various surgical interventions.

Tissue pieces were washed in PBS, then cut into pieces of 2x2 mm size, then put into an NH_4Cl solution, in order to hemolyze the blood cell rests. This was followed again by washing in PBS and the culture medium. The pieces were then placed in Petri dishes. The skin pieces were also covered by a glass slip. At last, M-199 with 20% FBS culture medium was pipetted on the tissue pieces. Culturing took place at 37°C, 5% CO_2 and 80% humidity. The culture medium was changed twice per week. As a rule, we obtained confluent cultures within 6 weeks. At this time the cell monolayer was removed by trypsin-EDTA treatment, and further

grown in culture flasks in M-199 with 10% FCS, until the required cell numbers were achieved.

The treatments of the cell cultures

Studies of the effects of $OH^{\bullet}$ free radicals were carried out by applying Fenton reactants. We added to the cultures Fe^{2+}-ADP complex and H_2O_2 generating this way $OH^{\bullet}$ free radicals. Final concentration of iron was 100 μM in the culture medium. The H_2O_2 was added to reach a final concentration of 55 μM to the primary cell cultures, the 3T3, and L929 cells, while it was 100 μM in case of K-562 cells.

Usually we applied $2x10^5$ cells/ml starting cell suspension, using one control and one Fenton-treated cell group for 96 hrs. The culture medium was changed after 48 hrs, and the Fenton treatment repeated. Ara-C treatment was also applied to a third group (1.8 μM final concentration).

The following methods have been applied on the cell cultures:

Viability test with Trypan-blue stain

Detection of hemoglobin by benzidine

Detection of hemoglobin histochemically by DAF (2,7- diaminofluorene)

Flow-cytometry to follow the differentiation of K562 cells

For this last method, cells were fixed in 70% ethanol, and diluted to the ideal cell density. Informations were collected from 10,000 cells in the FSC and SSC modes, and the necessary cell population was marked by applying digitalized “gates” to them.

Determination of the iron content of the culture media by atomic absorption spectrometry (AAS)

Iron was measured by means of the flame technique directly from the supernatant, after a deproteinization of the culture medium by 10% trichloracetic acid and centrifugation.

GAG-measurement by spectrophotometry after dimethylmethyene-blue (DMB) reaction

GAG histochemical staining by dimethylmethyene-blue (DMB) reaction

Detection of the Ki-67 proliferation antigene by immunohistochemistry

Fat staining with Oil red-O

Fat detection by Sudan IV. staining

Solubilization of the cells for enzyme measurements (SOD, catalase)

Measurement of superoxide-dismutase (SOD) activity in cell solubilizate

Measurement of catalase activity in cell solubilizate

Measurement of DNA content

Measurement of protein content

4. Results

Experiments with K562 cells, and their results

The K562 human erythroleukemia cell line grows unlimited under normal culture conditions, and displays spontaneous differentiation only in 3 % of the cells. When these cells differentiate, they stop growing and similarly to the normal red blood cells, they start to synthesize hemoglobin. We generated oxygen free radicals in the K562 cell cultures by adding Fenton reactants (Fe^{2+}ADP + hydrogen-peroxide). Measuring the iron content of the culture media (RPMI and M199) by ASS, we found 2.25 mg/l, i.e., 4.5 μM. Comparing to this starting value, the addition of Fenton reactant increased the iron content about 22-times.

Apart from the untreated control cells, we applied also a so-called positive control treatment with 1.8 μM 1-β-D-arabinofuranosylcytosine (Ara-C), known to induce the erythroid differentiation of these cells. Cell counting proved unanimously that both the Ara-C, and the Fenton-treatment decreased the cell growth in a statistically strongly significant manner, i.e., inhibits the cell proliferation. The accumulation of hemoglobin in the erythroleukemia cells can be detected by counting the benzidine positive cells in Bürker chamber. This way we could prove the differentiation inductive effect of the free radicals, as shown by the significant increase of the hemoglobin positive cells. However, the microscopic judgement of the positive cells may represent some difficulties. Therefore, we introduced the 2,7-diaminofluorene (DAF) staining for hemoglobin. This method has the following advantages: while the benzidine reaction interferes with the culture medium, first of all with the serum components of it, the DAF-staining is not disturbed by the presence of serum. This offers a possibility for the quick, simultaneous evaluation of multiple samples in multiwell plates, and in addition, the photometric determination of DAF-hemoglobin is about 80-times more sensitive than the benzidine-hemoglobin reaction. The use of this new compound

is also justified by the fact that benzidine is a proven mutagenic and carcinogenic compound, yet it is still generally used for the detection of hemoglobin in many laboratories.

Hemoglobin was demonstrated in K562 cells treated by Ara-C and Fenton reactants, using the DAF staining. It is conspicuous that the untreated cell populations display only a few DAF-positive cells, while the Ara-C treatment induced significantly the hemoglobin synthesis. Also the Fenton treatment resulted in numerous DAF-positive cells, proving that generation of OH· free radicals in the blast cell cultures stimulates the differentiation of the cells.

When studying the K562 cells by flow-cytometry, we found in two parallel experiments an unanimous increase of the cell size by the FSC records in both the Fenton- and Ara-C-treated cultures, measuring 10,000 cells per sample.

A conversion of the intracellular mass into a more compact structure (increase of granularity) have also been convincingly demonstrated in both of the treated cultures, as compared to the controls, as revealed by the SSC records. It seems to be evident, that the cytoplasm undergoes similar structural alterations also in the Fenton treated cells, which are known signs of cell differentiation, i.e., of the hemoglobin accumulation. These results represent further experimental evidences for the differentiation inductive effects of the OH· free radicals in the blast cells.

Experiments with human fibroblasts and blast-type tumor cells, and their results

Our studies were extended to primary human cell cultures, in order to find a basis for generalization of the observations obtained in mostly immortalized cell lines, regarding the effects of the free radicals. We grown fibroblasts for this purpose from the following tissues: non-EOP retrobulbar muscle, EOP retrobulbar muscle, non-EOP retrobulbar fat tissue, EOP retrobulbar fat tissue, abdominal skin.

In these cells we performed also the measurment of two enzyme activities (superoxide dismutase = SOD and catalase) which play a pivotal role in the formation and elimination of the oxygen free radicals. The results of these two enzyme measurements show an increased gene expression of these enzymes under the Fenton treatment. These results agree well with our previous

observations obtained on malignant cell lines (HL-60, K562, PC-12, SK-N-MC, etc.) in our laboratory.

In order to prove that under the influence of the free radical flux the cell proliferation is slowing down, we performed the histochemical demonstration of the Ki-67 proliferation antigen. We investigated the EOP and control cells after Fenton treatment. In both groups, about 40-50% of the untreated cells were positive for this antigen, while under the free radical flux, the proportion of the positive cells decreased to very low level, to several percent. These experiments have proven again that the first step in the differentiation induction by the free radicals is the slowing down of the cell proliferation rates.

The retobulbar fibroblasts of EOP patients are able to produce great amounts of GAG, and the extraordinary water-binding ability of them plays an important role in the mechanism of exophtalmus. We analyzed whether the in vitro GAG production of the retrobulbar fibroblasts is altered by the free radical treatment. The presence of GAG was demonstrated by 1,9-dimethylmethylene-blue DMB) stain, giving a bluish-violet metachromatic staining with the GAGs. After the 3-month-long treatments, the histochemical reaction was performed in situ, i.e., on the very same culture flasks where the cells were grown. The cells were fixed in the flasks, then the flasks were cut so that we could remove all the upper parts of it, obtaining this way a large “slide”, allowing us to perform the staining and covering of the cells very easily. In the untreated control cultures we observed only a minimal metachromatic DMB staining, while already a 1-week-long OH· flux considerably increased this metachromasy, i.e,, the GAG synthesis. This tendency was further increased by 1-month-long treatments, and at this time one could also observe various morphological changes of the cells. Newly, their spindle shape disappears and they become cells of multiple processes in various directions. These morphological changes become strongly evident after 3 month of treatment. This observation may be of importance in the understanding of the retrobulbar tissue growth, since most of the GAGs are able to swell even in the extracellular space.

For the quantitative determination of the GAG production of the cells, we performed also a spectrophotometric GAG measurement. This measurement also

confirmed that already a 1-week-long Fenton treatment results in a measurable increase of the GAG production in both cell populations studied. The parallel investigations all agreed in their tendency, with minimal quantitative differences.

In conclusion, both the histochemical and the photometric results supported the view that the retrobulbar fibroblasts are able to respond to the Fenton treatment with an increased GAG production in vitro, and the amount of produced GAGs is increasing in time.

Studies on the problems of fibroblast-preadipocyte transition

At the beginning, the EOP patients suffer from an autoimune disease resulting in a swelling of their retrobulbar tissues, leading to the formation of exophtalmus. In the maintenance of the exopthalmus, a particular significance is attributed to the hyperlasy of the retrobulbar fat tissue, which seems to be irreversible according to the present knowledge. Its causes and origin are unclear. The transformation of human orbital fibroblasts into preadipocytes has so far been studied only by using specific fat-cell producing protocols, e.g., addition of IBMX, dexamethason, inzulin, biotin, prostaglandins, TSH has been described as necessary factors of this process. The fibroblasts of skin origin were usually used as negative controls, assuming that they are unable for adipocyte transition anyway, even in the presence of the listed components.

When culturing retrobulbar and skin fibroblasts under normal circum-stances, Oil red O fat staining revealed that in these cells we can see significant staining intensities even without using the specific fat-inducing protocols, and furthermore, the staining instensities were strongly dependent on the actual surface where the cells were kept. In order to clarify this problem, we grown cells on 5 qualitatively different surfaces, and performed the stainings of them on the same “slides”.

In addition to the untreated primary orbital and skin fibroblasts, we studied also NIH/3T3 and L929 cell lines, the spontaneous preadipocyte transition so far has not been described. To control the Oil red O staining, we used also Sudan IV staining. The main observation was that on glass surface, or collagen coated glass surface all these cells displayed an intense fat-staining, while on the plastic surfaces the staining intensity was almost negligible.

Our observations led us to the conclusion that from the point of view of fibroblast-preadipocyte studies, it is of crucial importance how the culture surface quality may influence the fat-staining. It is possible that the quality of the surface

(e.g., electrostatic charging) acts on the cells, and as an inhibitor of lipid accumulation, exerts its effect through specific processes. The surface quality may have an influence on both the lipid accumulation, and also on the lipid staining. Although the exact explanation of these phenomena is still missing, their existence should be considered. In the light of these data, one has to reconsider numerous experimental findings, regarding the fibroblast-preadipocyte differentiation procedure. It is perhaps not an exaggeration to claim that according to our opinion, all sorts of fibroblasts are able to fat accumulation, i.e., all fibroblasts may be considered practically as preadipocytes which are able to become adipocytes under proper circumstances and signals. This concept may allow some new interpretations. Namely, numerous clinical problems, like the exophtalmus in endocrin ophtal-mopathy, or even the actually very widespread disease, the obesity, may be much better understood.

Since the fat accumulation is a differentiation marker phenomenon, we studied also the effects of OH· free radicals on the orbital and skin fibroblasts. Under the Fenton treatment we observed an intense fat accumulation in both the EOP and control fibroblasts. This observation well agrees with the previous ones, i.e., that the free radicals induce differentiation phenomena in all cell types. In other words, the free radical flux induces differentiation also in the normal blast cells, quite similarly, as in the immortalized cell lines. The differentiation signs may be quite variable, e.g., hemoglobin, GAG, triglyceride, et., synthesis, processes which are present in the undifferentiated blast cells only to a very low extent. In addition, our results point out also to the fact that the field of fat differentiation may still suffer from the need of revisions at various sites, since very simple factors, like the quality of the culturing surface may be of great influence on the experimental results, and also on the interpretations.

5.Summary

The differentiation inducing effect of OH• free radicals has been investi-gated using various models and techniques. Differentiation was induced in K562 erythroleukemia cells by Fenton reaction, generating OH• free radicals. To detect hemoglobin, the benzidine has been replaced by diaminofuorene (DAF). These cells displayed an intense hemoglobin synthesis under the free radical treatment by DAF staining, and in addition, applying flow-cytometry, we observed an increase of cell size and compactness of the cytoplasm. In the fibroblasts grown from retrobulbar muscle and fat tissues of EOP patients, 1-week, 1- and 3-month-long treatments with OH• free radicals induced an increased GAG production. This is an important observation, because the increased GAG production, and the high water-binding ability of these compo-unds are one of the main reasons for the exophtalmus in EOP. SOD and catalase activities of these primary fibroblasts were also determined under the effect of free radical flux. The gene expression of both enzymes proved to be stimulated by the free radicals, indicating an intensified radical turnover, which may play an important general role in the cell differentiation mechanisms. Applying the histochemical demonstration of the Ki-67 proliferation antigen, a significant decrease of the proliferative activity was observed in both the EOP and control fibroblasts after Fenton treatment, which is the first step of cell differentiation. Regarding the causes of retrobulbar fat accumulation in EOP, we obtained information by studying the fibroblast-preadipocyte transition, since the adipocyte differentiation may play an important role in this process. Oil red O and Sudán IV fat stainings were performed on primary human retrobulbar and skin fibroblasts, as well as on 2 fibroblast-type, immortalized cell lines, L929 and 3T3. Ot has been observed that the fat stainings depend on the quality of culture surface, being different on glass and plastic surfaces. On glass surfaces one could see very intense fat-stainings without using any fat-differentiating agents. Our results show that all kinds of fibroblasts are able to spontaneous fat accumulation, and if proper signals are present, they may become fat cells. An increase of the radical flux induced differentiation phenomena in all so far studied blast-type cells, showing that it is hardly possible to maintain the free radical theory of aging in its original form, according to which the oxygen free radicals are only harmful byproducts.