

## MODULATION OF ELASTIN SYNTHESIS IN PRIMARY CULTURES OF HUMAN FIBROBLASTS

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**ABSTRACT.** The *novo* synthesis of elastic fibers proceeds in adult tissues at a very low rate and with deficiencies unlike the perfect remodelling of elastic fibers observed after an injury in embryonic tissues. In this study, the elastin synthesis in two fibroblast cultures obtained from normal adult human skin (AF) and human embryos (EF) and its modulation in the presence of dexamethasone were analyzed. A direct ELISA method with bovine  $\alpha$ -elastin as standard and a mouse monoclonal antibody against  $\alpha$ -elastin was employed to quantify soluble elastin in these two cell populations. Our results indicated that in AF culture the amount of synthesized elastin was four times smaller than in EF. After dexamethasone stimulation, in the culture medium from AF culture, the values for elastin raised 2-3 times, being close to those from unstimulated EF. The maximum stimulation was observed at a final concentration of  $10^{-7}$  M dexamethasone, after 48 hours of cultivation. On the other hand, cell growth was slowed down in the presence of dexamethasone. Calculating the relative stimulation of elastin biosynthesis in AF an increase of 190% and 265% in 24h and 48 h, respectively, was observed.

**KEYWORDS.** elastin, tropoelastin, ELISA, fibroblast cultures

### INTRODUCTION

Elastin, the main protein responsible for the resilience of some vital tissues like blood vessels, lungs and skin is characterized by a turn-over equal to the organism lifetime [1]. However, progressive degradation of elastic fibers and the loss of their physical properties and biological functions due to the lipid and calcium fixation leads to different pathologies of elastic connective tissues.

While collagen fibers can be synthesized whenever necessary, the synthesis of elastic fibers takes place only in the last gestation period and early after birth [2]. The

neosynthesis of elastic fibers in adult tissues has a very low rate and is imperfect unlike the perfect remodelling of elastic tissues in embryonic tissues [3]. *In vitro* modulation studies on fibroblasts from adult tissues reported that only small amounts of biosynthesized soluble elastin could be obtained [4, 5]. In an elastase-emphysema model on adult hamsters elastogenesis could be reactivated and the elastin content in lung tissue was restored after 2-3 months [6].

The aims of this study were (a) to compare two fibroblast cultures from normal adult human skin (AF) and human embryos (EF) and b) to modulate elastin biosynthesis in AF and EF in the presence of a glucocorticoid (dexamethasone).

## MATERIAL AND METHODS

**Materials.** Mouse monoclonal antibody to  $\alpha$ -elastin, peroxidase-labelled rabbit anti-mouse IgG (RAM-HRP), 3,3',5,5'-tetramethylbenzidine (TMB), dexamethasone, insoluble elastin and albumin fr. V were all purchased from Sigma. Microtiter plates were from Nunc.

### **Methods.**

**Cell culture.** Adult dermal fibroblasts (AF) were isolated from human skin using the outgrowth technique. Tissue fragments were surgically removed from donors, cut in 2 mm<sup>2</sup> pieces, placed in Dulbecco's modified Eagle's medium (DMEM), containing 5% bovine fetal serum, HEPES buffer and antibiotics, and maintained in 5% CO<sub>2</sub> humidified air, at 37<sup>0</sup>C. Unattached explants were moved to other Petri dishes until all fragments attached and the cell growth was stabilized. Cells obtained from explants were trypsinized and cultivated in 100 mm<sup>2</sup> Petri dishes. Embryonic fibroblasts (EF) were isolated by trypsinization of normal embryos from miscarriages.

Culture medium was changed twice a week. First and second passage of AF culture and 9<sup>th</sup> and 10<sup>th</sup> passage of EF culture were used in the experiments. After each experiment, the number of viable cells was determined.

**Direct-ELISA.** The protocol established for this study is based on the direct-ELISA technique which allows the analysis of serial samples taken from the culture medium [7]. We optimized the method for the assessment of tropoelastin biosynthesized in primary cultures of AF and EF, as described earlier [8]. In parallel experiments, confluent cultures of AF and EF were cultivated in fresh DMEM with 10<sup>-9</sup>, 10<sup>-8</sup> and 10<sup>-7</sup> M final concentration of dexamethasone. After 72 hours, in samples from the conditioned medium, tropoelastin was quantified by the same protocol. All measurements were made in triplicate.

**Light microscopy.** Cell morphology was examined daily at a Nikon inverted microscope. Also, slides with AF and EF were removed from the cultures, washed in phosphate buffer saline pH 7.5, for 3 minutes, fixed in methanol, for 5 minutes and stained with Hematoxylin-Eosin, for 20 minutes. Then, the slides were washed thoroughly with distilled water, dried, mounted in Canada Balm and visualized at a Leitz microscope.

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## RESULTS

### *Cell morphology*

In our study, the cultured fibroblasts isolated from human adult skin showed a typical spindle-shaped morphology, with a bipolar appearance, a homogeneous cytoplasm and bulky nuclei in which a variable number of nucleoli could be observed (fig. 1A). Also, we noticed that the cell spread over a large surface and the dense fiber areas were present. Fibroblasts from embryonic tissue showed a rounded morphology with short cytoplasmic extensions (fig. 1B).

These observations indicated that there were some morphological differences between AF and EF in primary cultures.

Multiple passages of AF cultures induced alterations in the cellular phenotype due to prolonged cultivation on the synthetic support. Many degenerated cells with pycnotic nuclei, micronuclei and nuclear fragmentation specific to apoptosis were observed (fig. 2). Because these alterations could lead to artefacts, in our experiments only cells obtained in the first two passages were used. On the other hand, EF proliferated after reaching confluence forming multiple layers.

### *Optimization of elastin synthesis*

The cellular growth was optimal in the presence of 10% fetal bovine serum but some compounds from the serum could interfere with the immunological technique used in the determination of elastin synthesis. Thus, only 5% fetal bovine serum, enough for cell development was added in the culture medium.

We carried out a comparative investigation of tropoelastin biosynthesis, quantified by a direct ELISA method, in embryonic and adult dermal fibroblast cultures. We used the first and second passages of the AF primary cultures to avoid the rapid decrease in the tropoelastin synthesis that occurs with serial subculture [9].

Because the elastin production in AF is relatively low compared to other elastogenic cells, we stimulated the cells using a synthetic steroid hormone, dexamethasone. AF were cultivated in the presence of dexamethasone in final concentrations of  $10^{-9}$ ,  $10^{-8}$  and  $10^{-7}$  M, respectively, and, after 48 h, the synthesized elastin by ELISA was quantified (fig. 3). A concentration of  $10^{-7}$  M dexamethasone induced an aprox. two fold increase of the biosynthesized elastin compared to the control (AF cultured in the absence of dexamethasone).

The dexamethasone stimulation of elastin synthesis in AF for 72 hours was measured daily and was shown in figure 4. The obtained curve had three segments: a steep slope in the first day of cultivation (24 h) corresponding to a significant increase in elastin synthesis, a plateau during the next day (48 h) and a slow decrease in the last day (72 h). A dexamethasone exposure of minimum 24 hours is required to induce stimulation of elastin synthesis in AF.

Next we compared the levels of elastin synthesis in samples from the culture medium of AF and EF incubated or not with  $10^{-7}$  M dexamethasone for 48 hours (fig. 5). The soluble elastin quantity in AF had lower values than in EF. After the addition of dexamethasone to a final concentration of  $10^{-7}$  M in the culture medium the value of synthesized tropoelastin increased to similar values recorded in unstimulated EF.

The relative stimulation of elastin synthesis was calculated by dividing the elastin concentration measured in the presence and in the absence of the hormone (table 1). The value of the relative stimulation in EF was smaller than in AF.

Steroid hormones have been shown to decrease the proliferation rate of many cell types [10]. The effect of dexamethasone on AF was determined after cultivating the cells in culture medium in the absence or presence of  $10^{-7}$  M dexamethasone for 3 days. The population doubling time of cells grown in the presence of dexamethasone was slower than that of cells grown without the hormone (fig. 6).

## DISCUSSION

The fetal wounds can heal without contraction and scar formation [11]. Still, the mechanisms underlying this process are not understood. It was proposed that fibroblasts from fetal skin are different from those from adult skin.

For better understanding of elastin biosynthesis in AF, we compared in this study the parameters implied in this process in two fibroblast cultures: one from human adult skin and one from human embryos. Unlike other *in vitro* studies using EF for the biosynthesis of tropoelastin [12] we established the cultivation parameters for AF to analyze the soluble elastin production and its modulating factors.

We chose to do these experiments using fibroblast cultures because, regardless of the source from which were isolated, these cells secrete in the culture medium 90% of tropoelastin synthesized and no cross-linking process for insoluble elastin formation was observed [5]. Moreover, the elastin phenotype is much more stable than in most other cell types. In other studies, fibroblasts from bovine ligamentum nuchae were cultured because they secrete a large quantity of soluble elastin [13]. In smooth muscle cells from rat aorta the secreted tropoelastin was transformed in insoluble cross-linked elastin [14]. The chondrocytes secreted a large quantity of tropoelastin in the medium but when it accumulated, it associated with the cellular layer and after confluence formed insoluble elastin [9].

The ELISA-based method was used because it allowed elastin quantification direct in culture medium samples and avoided the isolation and purification of tropoelastin by immunoprecipitation, procedures that led to semnificative loss of tropoelastin (aprox. 75%) due to enzymatic proteolysis and adsorbtion to the dialysis membrane and extraction vessel walls [7].

The effect of glucocorticoids on cell cultures from connective tissues is an inhibitory one leading to a decrease in the biosynthesis of matrix components: glycosaminoglycans, collagen, hyaluronic acid [10]. However, they have a stimulatory effect on elastin biosynthesis *in vivo* and *in vitro*, regulating the elastogenic differentiation during development and modulating the elastin gene expression [15]. The necessity for stimulating the elastin biosynthesis in dermal fibroblasts in culture was indicated by studies at molecular level which demonstrated an mRNA level for tropoelastin in adult and fetal cells 3-4 times smaller than in tissues. These results demonstrated a progressive decreased elastin biosynthesis in cell culture [13].

Our study indicated a smaller relative stimulation of EF in the presence of dexamethasone compared to AF. This result was in contradiction with other steroid stimulation studies made on ligamentum nuchae fibroblast cultures [16]. A possible explanation could be the ageing of the cultivated cells after repeated passages, concomitantly to a reduction of their biosynthetic capacity.

### CONCLUSION

Dexamethasone had a modulating effect on human adult fibroblast in culture. A concentration of  $10^{-7}$  M dexamethasone induced a stimulation of elastin biosynthesis by 190% in 24 hours and 265% in 48 hours.

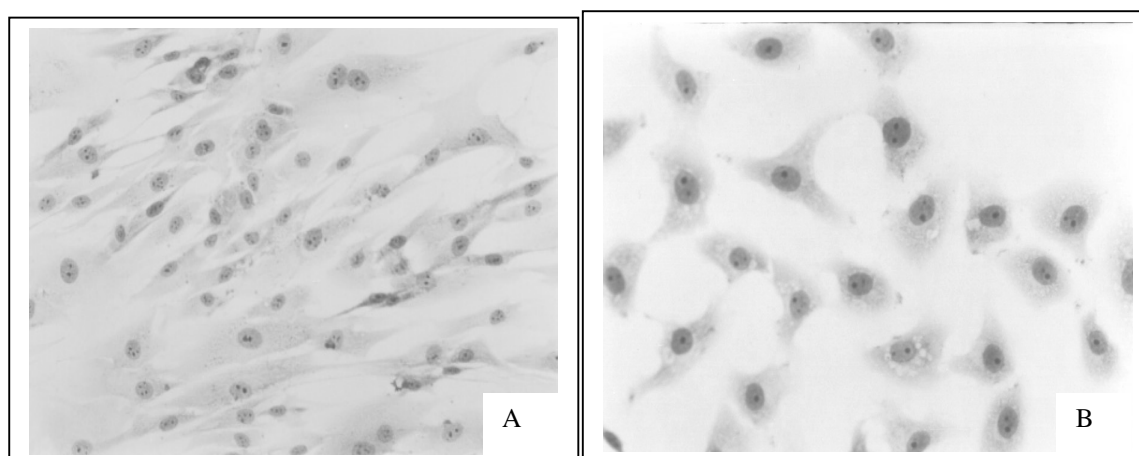
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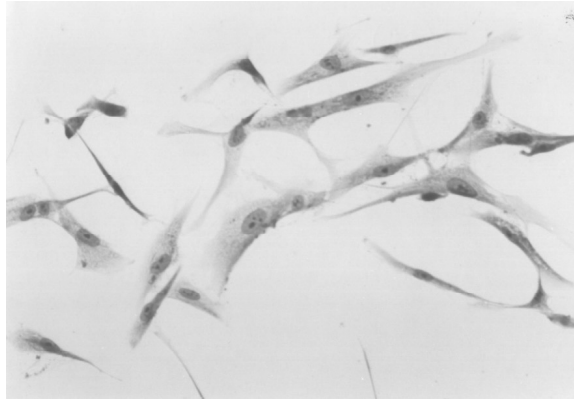
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**Table 1** – The effect of dexamethasone on elastin biosynthesis in AF and EF

<b>Cultivation time (hours)</b>	<b>Soluble elastin synthesis (ng soluble elastin equivalents/10<sup>6</sup> cells)</b>	<b>Relative stimulation (%)</b>
24	26	190
48	34,5	265

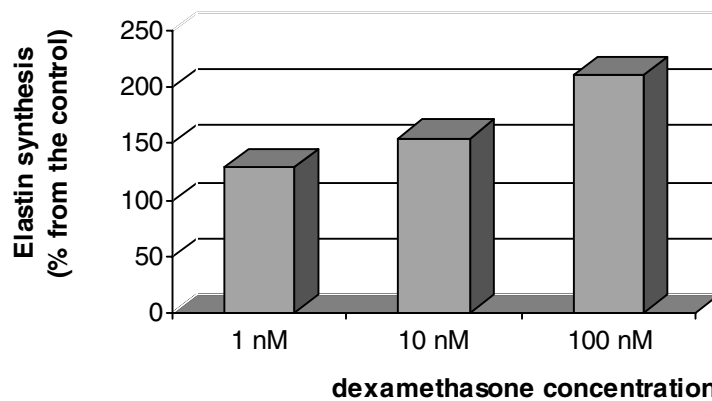


**Figure 1** – Light micrographs of fibroblast cultures from A. human adult skin (AF) and B. human embryos (EF). (Hematoxylin-Eosin staining) (A, x160; B, x250) AF showed in culture a typical bipolar spindle-shaped morphology. EF had a rounded morphology.

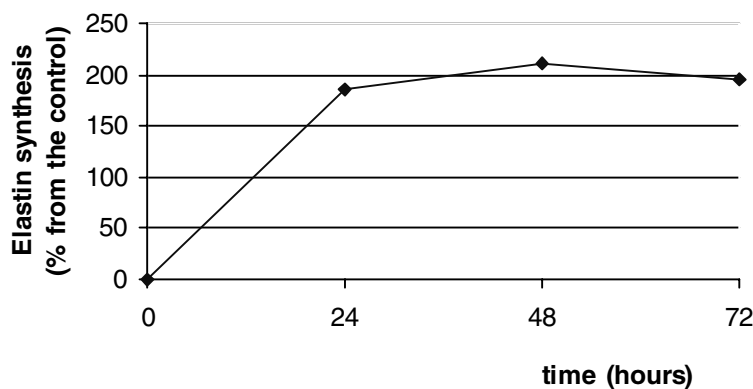


**Figure 2** – Light micrograph of human dermal fibroblasts after several passages (Hematoxylin-Eosin staining, x250).

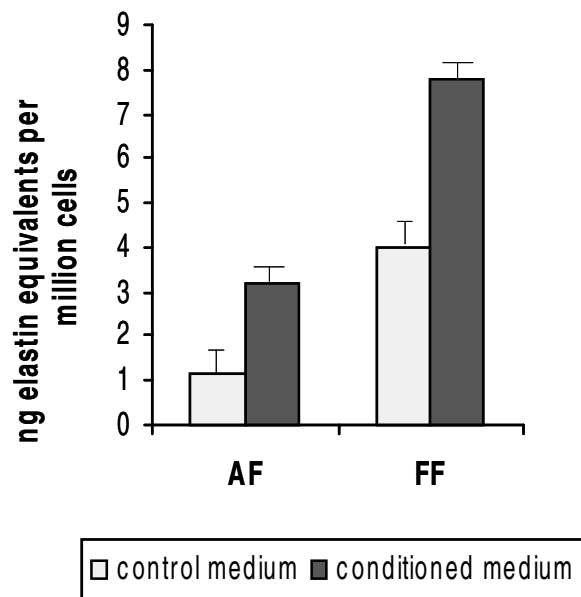
It were remarked many degenerated cells with pycnotic nuclei, micronuclei and nuclear fragmentation.



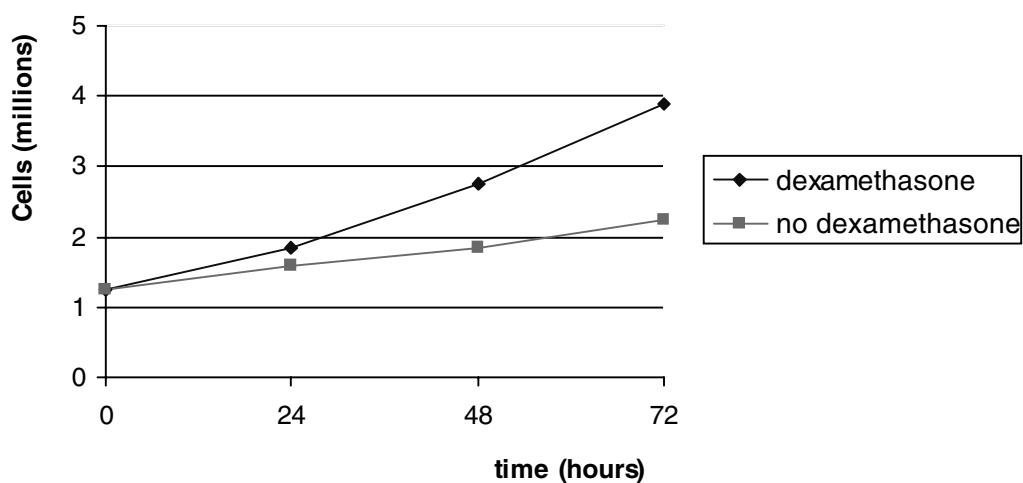
**Figure 3** - Elastin synthesis in AF cultivated in the presence of different final concentrations of dexamethasone, for 48 hours, determined by direct ELISA.



**Figure 4** - Elastin synthesis in AF cultivated in the presence of  $10^{-7}$  M dexamethasone, for 72 hours, determined daily by direct ELISA.



**Figure 5** – Elastin quantification in the culture media of AF and EF in the presence and absence of  $10^{-7}$  M dexamethasone, by direct ELISA. The values represented the mean value  $\pm$  variation for three identical samples.



**Figure 6** – Kinetics of cell growth in the presence and absence of  $10^{-7}$  M dexamethasone. It was remarked that cells grown slower in the presence of the hormone.