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Cu/Zn superoxide dismutase modulates phenotypic changes in cultured fibroblasts from human skin with chronic radiotherapy damage

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Abstract

Purpose: As we previously observed that bovine liposomal Cu/Zn SOD (LipSOD) reduces cutaneous radiation-induced fibrosis (RIF) in human therapeutic assays the mechanisms involved were investigated here by an in vitro study of the LipSOD effects on cellular antioxidant metabolism and regulation of matrix degradation.

Methods: Primary cultures of human fibroblasts harvested from normal or RIF skin were treated with various doses of LipSOD. Catalase, Cu/Zn and Mn SOD endogenous cell enzyme activities and protein amounts were assayed by polyacrylamide gel electrophoresis and western blotting. Gene expressions of tissue inhibitor of metalloproteinases (TIMP) and TGF-β1 was investigated by northern blot analysis.

Results: A deficiency of endogenous Mn SOD, considered to favour cell proliferation, was observed in cultured RIF cell. The present study showed that bovine Cu/Zn SOD entered the cells. Exposure to LipSOD (a) enhanced endogenous Mn SOD activity and protein level, without changes of endogenous Cu/Zn SOD and catalase, and (b) significantly reduced TIMP and TGF-β1 gene expression, in RIF cells. No changes in these parameters were noted in treated control skin fibroblasts.

Conclusion: Modulation of RIF skin fibroblasts by LipSOD seems effective via indirect endogenous Mn SOD activation, which might explain the cell phenotype reversion observed. TIMP reduction accounts for the elimination of collagenase activity inhibition and the subsequent digestion of excess extracellular matrix deposition, as well as RIF reversibility in vivo. The reduction of TGF-β1 expression might explain the breaking of maintaining fibrotic cell activation connected with this growth factor.

Keywords: Radiation-induced fibrosis; Superoxide dismutase; Transforming growth factor-β1; Tissue inhibitor of metalloproteinases; Cultured fibroblasts

1. Introduction

Superficial radiation-induced fibrosis (RIF), a late effect of therapeutic or accidental high-dose irradiation, is well known in clinical practice. After an asymptomatic cutaneous prefibrotic phase, it is characterized by local inflammation and swelling. With time, this early active RIF undergoes step-wise aggravation and becomes old constituted fibrosis, often clinically associated with superficial atrophy and gradual destruction of cutaneous and subcutaneous normal tissues. After local aggressive physicochemical treatment, or in predisposed patients, the old RIF may sometimes lead to late radiation necrosis [10,11].

The postulate of the irreversibility of established fibrosis was challenged by clinical observations in patients, in whom it was possible to reduce, significantly and durably, chronic radiotherapy damages using exogenous Cu/Zn superoxide dismutase (SOD) in a liposomal form (LipSOD) [12]. The therapeutic effect was a mean regression of 60% of RIF surface area in two thirds of patients. This result was subsequently reproduced under experimental conditions, in our pig model simulating accidental overexposure [21], where a normalized tissue vascularization and histological signs of muscular regeneration were observed [20]. However, although the role of SOD is the dismutation of superoxide radicals, many questions remained unanswered concerning the mechanisms by which SOD reverses the fibrotic process and permits the replacement of scar tissue, characterized by a dense and hyalinized matrix, poorly vascularized with fewer fibroblasts [7,9,33], by normal tissue. Therefore, it was necessary to study these mechanisms in an in vitro human model. In vitro, in monolayer cell culture, some fibrotic cells exhibited an enhanced growth

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potential, whereas others displayed either the same or a reduced potential as the control fibroblasts. It's the reason why we have developed a human fibroblast protocol from samples of skin treated by radiotherapy 6 months to 9 years before [13]. This culture was possible and reproducible, but limited because of a high rate of spontaneous cell death. The 'surviving RIF cells' displayed major signs of exhaustion with a reduced proliferation ability, unchanged tissue inhibitor of metalloproteinases (TIMP) and transforming growth factor beta (TGF-β1) antiproteasic activity. and a reduction of Mn SOD and catalase antioxidant activities [13].

We postulated that the mechanisms of LipSOD action in vivo might be an activation of depleted endogenous antioxidant metabolism, a key regulator of cell proliferation, and an activation of the proteases activities through the down regulation of TIMP and IGF-β1 expression, then the matrix degradation. Most questions concerning the mechanisms governing this in vivo reversion of the fibrotic tissue after treatment with LipSOD are still unanswered. To explore the possible actions of LipSOD on the cellular antioxidant metabolism and the regulation of matrix degradation, we studied the effects of LipSOD on cultured fibroblasts from human old constituted RIF tissue.

2. Patients and methods

2.1. Population

Five irradiated patients, whose mean age was 59 years (range: 46–75) and who had been treated for breast cancer (three women) or head and neck cancer (two men), were given 60–70 Gy as postoperative adjuvant irradiation [13]. Surgical skin specimens, free of evolutive cancer were taken 6 and 9 months after irradiation, and 2.5, 5 and 9 years thereafter. The samples were labeled RIF 1 to RIF 5 and were excised as part of programmed surgery which included a curative procedure approved by our Ethics Staff. These samples were defined by hardened and thick fibrotic skin, without local inflammation. Five control dermis specimens were obtained from patients whose mean age was 49 years (range: 39–65), either from the neck or upper chest skin of the same patient (two cases) or from healthy control subjects (three cases).

2.2. Cell culture

Enzymatic separation was impossible, because of the samples size. Culture were initiated from explants placed in six-well dishes in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal calf serum (FCS). Fibroblasts migrated out of the explant, according to the model described previously [13]. There was no delay in the outgrowth of cells derived from RIF compared to control skin outgrowth (4–10 days in both groups). Samples were removed when the growing cells had formed a complete ring. The mean period until cells reached half confluence was fairly constant in time: i.e. 22 days (20–28) for controls versus 25 days (20–27) for RIF cells. However, this cell growth was not regular in the space: control cells grew forming homogeneous continuous ring round the explant, whereas RIF cell proliferation was heterogeneous with spindle shape cells with a normal appearance far from the explant and small discoid cells round the explant [13].

After this primary culture, the fibroblasts were harvested by enzymatic detachment and cultured under standard conditions, in a medium containing DMEM, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (FCS), 4 mM glutamine. Briefly, cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, detached with trypsin, passaged weekly. counted in a Malassez chamber and stored in suspension, in 10% DMSO at −180°C in liquid nitrogen. They were seeded in plastic flasks (Nunc) at 4×10^5 cells/cm^2 in single-layer cultures and passaged at confluence. At each passage, the cells were counted with a hemocytometer (Malassez), and for each data point of the ten cell lines, we took in account the mean results of three flasks.

Control dermis (CD) cells displayed exponential growth kinetics from passages 1 to 4, and a constant number $96.10^3 \pm 27.10^3$ of cells/cm^2 at confluence, which was reached after a mean period of 6.4 ± 1.5 days. CD cells included a small proportion of floating cells (mean 1% in passage 2) and this proportion remained constant. In contrast, most RIF cells exhibited a short finite lifespan in culture: the kinetics of RIF cell growth tended to form a plateau. In passages 1 to 3, all the cultures of the RIF cell lines were characterized by a large proportion of cell death. The proliferative capacity of the surviving RIF cells, which quickly entered senescence, declined gradually. Under the usual culture conditions, the five RIF lines needed an average of 8.5 ± 3.2 days to reach confluence. Similarly, cell density dropped from $87.10^3 \pm 43.10^3$ cell/cm^2 at passage 1, to $40.10^3 \pm 10.10^3$ cell/cm^2 at passage 3 [13]. Cells were cultured until a number of cells sufficient to perform the studies was obtained for each cell line, that is between passage 4–5.

2.3. LipSOD incubations

At confluence, cultured cells at passages 4 and 5 were incubated in medium with low FCS concentration (1–2%) and modulated with LipSOD. In the form of powder 5.63 mg of LipSOD was reconstituted in 2 ml of water, i.e. 10228 U of SOD/ml. As the endogenous Cu/Zn cellular SOD is estimated to be 1 μg/10^6 fibroblasts [29], we used 76 U LipSOD/10^6 fibroblasts, which corresponds approximately to six times the endogenous level and is referred to as the standard dose in the text. Toxicity tests were carried out between four to ten times the standard dose (68 and 240 μg LipSOD/25 cm^2, respectively).

2.4. Enzyme and protein assays

At confluence, cultured human fibroblasts at passages 4–5 were exposed to a standard dose of LipSOD and incubated for 20 h at 37°C. At the end of incubation, the culture medium was collected, and cells were washed twice in PBS. Cells were then scraped off the flasks in 10 mM Tris HCl, pH 7.5, 0.1% Triton X-100 and proteins were extracted by successive freezing and thawing. Enzymatic measurements and western blot assays were performed on the 17 000 × *g* supernatant. To determine endogenous Cu/Zn SOD and Mn SOD activities, 40 μg of each cell extract was loaded onto polyacrylamide gel consisting of 10% running gel and 4% stacking gel. Electrophoresis was performed at 190 V in Tris-glycine buffer, pH 8.6. The gel was stained according to Beauchamp and Fridovich. SOD appeared as colorless bands against a blue background. Quantification of Cu/Zn SOD, Mn SOD and catalase proteins was performed by Western blot as previously described [6]. The antibodies were obtained from Valbiotech (France). Detection was performed using Amersham kit Streptavidine-Phosphatase alcaline.

2.5. RNA isolation and northern blot analysis

At confluence, cultured human fibroblasts at passages 4–5 were exposed to a standard dose of LipSOD and incubated for 20 h at 37°C. The culture medium was carefully eliminated. Total RNA was extracted with 4 M guanidium isothiocyanate solution, isolated by the acid-phenol-chloroform method described by Chomczynski and Sacchi [8] and quantified by its absorbance at 260 nm. The absence of RNA degradation was checked by electrophoresis with ethidium bromide. For northern blotting, total RNA was separated in 1% agarose gels containing 2.2 M formaldehyde and transferred onto Nytran filters (Schleicher and Schuell). A 0.24–9.5 kb RNA ladder (BLR) was used as a size marker. Filters were crosslinked by ultraviolet. They were then hybridized at 65°C overnight to the SacI/PvuII insert of the TGF-β1 cDNA porcine probe pTGF-β33, which was labeled with P32 dCTP by random priming (megaprime DNA labeling kit, Amersham) according to our previously published method [20]. Blots were washed in 1 × SSC and 0.1% SDS at 55°C and then exposed at −70°C using intensifying screens. The same blots were washed and hybridized, first to a cDNA probe specific for the 18S fraction of mouse ribosomal RNA, which was used as internal control of RNA loading, and then to the EcoRI/EcoRI insert of a specific anticollagenase cDNA human probe coding for TIMP (Synergen, CO). Autoradiograms were quantified by scanning with a laser densitometer.

2.6. Statistical methods

Northern-blot quantifications were expressed as mean ± SD. The unpaired bilateral *t*-test and Wilcoxon test were used to compare the different groups. *P*-values of less than 0.05 were considered significant. Gels activities for SOD and western-blots were analyzed using the NIH Image software (NIH, Bethesda, MD).

3. Results

3.1. Antioxidant cell metabolism

3.1.1. Untreated cells

Control dermis fibroblasts exhibited higher level of endogenous Mn SOD than Cu/Zn activity (Fig. 1A). As previously described [13], endogenous Mn SOD activity was reduced in RIF cells as compared to control dermis fibroblasts, whereas no change was observed for Cu/Zn SOD activity (Fig. 1A). The amount of catalase protein dropped drastically in RIF cells with a 5-fold reduction factor (Fig. 2C).

3.1.2. LipSOD modulation

Treatment of cultures with a LipSOD concentration four times the standard one was very badly tolerated: we observed cell distress characterized by membranous swelling, without evident mortality. The metabolic analysis performed after a standard 76 U/10^6 cell LipSOD concentration showed that bovine Cu/Zn SOD penetrated into the cells (Fig. 2B). Endogenous Mn SOD activity remained unchanged in control dermis, whereas it increased on the

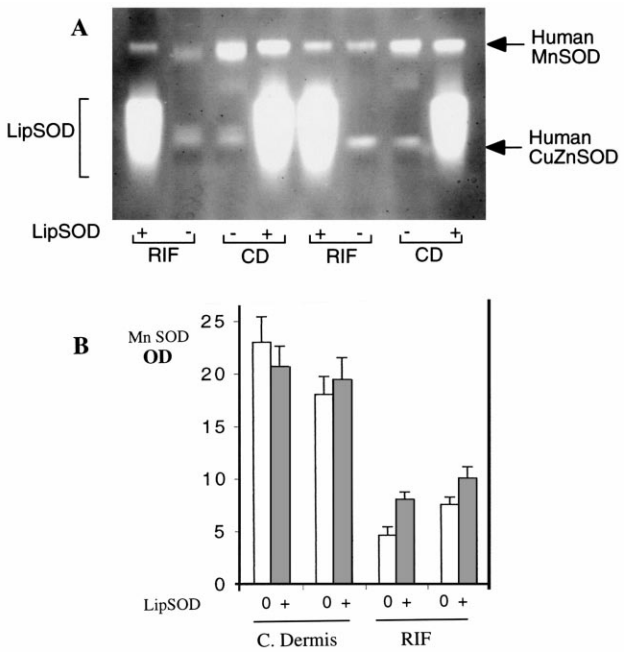

Fig. 1. Antioxidant metabolism of cultured fibroblasts. (A) Gel showing the enzymatic activities of endogenous Mn and Cu/Zn SOD in 40 μg of protein extract from two independent control dermis (CD) and two radiation-induced fibrosis (RIF) samples, treated (+) or untreated (−) for 20 h with 76 U/10^6 cells of LipSOD. (B) Histogram results of Mn SOD activity obtained after densitometric analysis (OD) performed on three different gels activity for these samples.

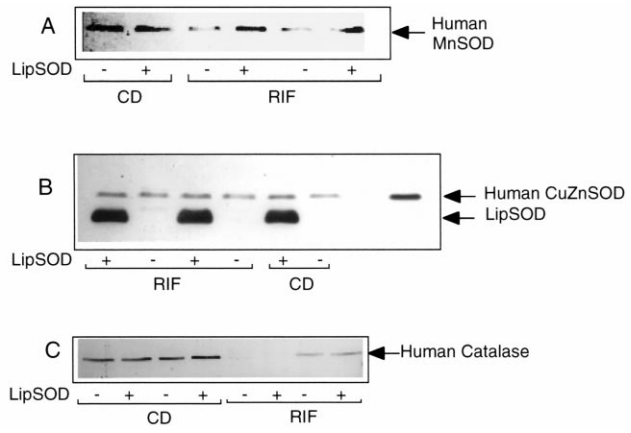

Fig. 2. Antioxidant metabolism of cultured fibroblasts. Western-blots for (A) Mn SOD, (B) Cu/Zn SOD and (C) catalase from 20 μg of protein ectracts obtained from control dermis (CD) and two independent radiation-induced fibrosis (RIF). Untreated cultures (−), and cultures treated (+) for 20 h with 76 U/10^6 cells of LipSOD.

average by a factor of 1.5 in the RIF cells (Fig. 1B) ($P < 0.01$). This result was confirmed at the level of Mn SOD protein amount (Fig. 2A). LipSOD modulation had no effect on the amounts of Cu/Zn or catalase proteins (Fig. 2B,C).

3.2. TGF-β1 and regulation of matrix degradation

3.2.1. Untreated cells (Figs. 3 and 4)

The amounts of TGF-β1 and TIMP gene expression in RIF cells were slightly but non-significantly smaller than in control dermis: the relative OD for TIMP/18S was 1.0 ± 0.03 in control dermis vs. 0.55 ± 0.03 in RIF cells ($P = 0.05$), and for TGF-β1/18S was 1.40 ± 0.70 in control dermis versus 0.93 ± 0.40 in RIF cells (n.s.), after quantitative analysis of the northern blots.

3.2.2. LipSOD modulation (Figs. 3 and 4)

After exposure to LipSOD, control dermis fibroblasts exhibited no change in their TIMP or TGF-β1 levels of gene expression. On the other hand, in RIF cells, TIMP and TGF-β1 RNA levels diminished 5- and 3-fold, respectively: the relative OD for TIMP/18S was 1.13 ± 0.35 in control dermis versus 0.24 ± 0.15 in RIF cells ($P < 0.001$). and for TGF-β1/18S was 1.86 ± 0.70 in control dermis vs. 0.64 ± 0.20 in RIF cells ($P < 0.01$), after quantitative analysis of the northern blots.

4. Discussion

In this in vitro study, fibroblasts from human superficial RIF tissue, were modulated by liposomal Cu/Zn SOD in an attempt to understand the mechanisms by which LipSOD leads to the regression of fibrosis in vivo. However, reference studies with non-vectorized SOD and with the liposome vector alone could not be carried out because the available amounts of irradiated human fibrous tissue were insufficient. Consequently, we were unable to test whether cytotoxic properties of high concentrations of LipSOD were attributable either to the high SOD concentration or to an excess of liposomes.

4.1. LipSOD modulation of the antioxidant cell metabolism

We showed that at moderate concentrations, exogenous bovine Cu/Zn SOD entered both control dermis and RIF cells, thus confirming the results of previous studies that demonstrated labeled SOD penetration of erythrocytes

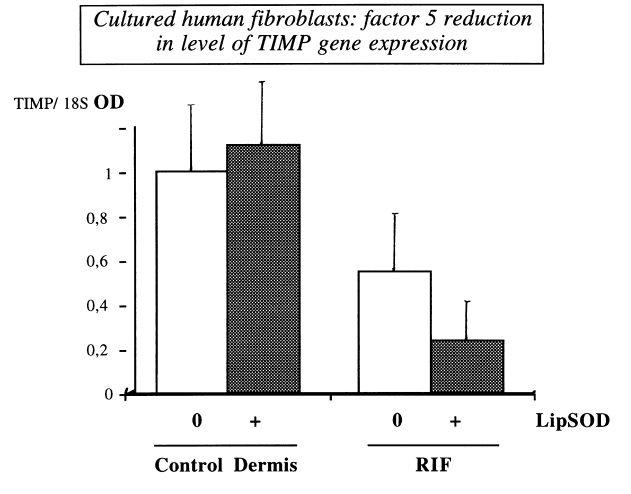

Fig. 3. Regulation of matrix degradation in cultured fibroblasts. Quantification by northern blot analysis of the TIMP gene expression, from control dermis (CD) or RIF cells total RNAs. Modulation by 20 h of cell exposure to 76 U/10^6 cells of LipSOD (+) with histogram showing optical densities (OD).

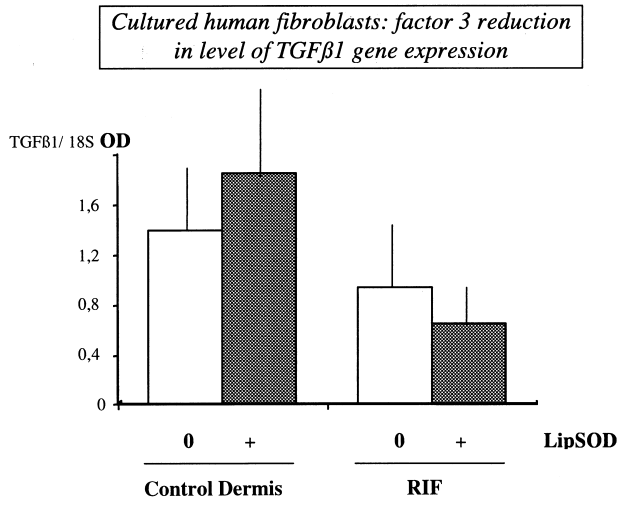

Fig. 4. TGF-β1 regulation in cultured fibroblasts. Quantification by northern blot analysis of the TGF-β1 gene expression, from control dermis (CD) or RIF cells total RNAs. Modulation by 20 h of cell exposure to 76 U/10^6 cells of LipSOD (+) with histogram showing optical densities (OD).

[30]. Incubation with LipSOD increased the amount of endogenous Mn SOD that might help to normalize the fibroblast phenotype. In this human cell culture, part of RIF cells exhibited a senescent phenotype and finally detached and floated in the culture medium. The 'surviving cells' displayed some characteristics of the myofibroblast phenotype with secreted matrix deposit which accumulated on cellular aggregates and exhibition of intense contractile activity [13]. These 'surviving' cells are characterized by the presence of significantly reduced amounts of Mn SOD and catalase proteins and activities, with unchanged endogenous Cu/Zn SOD ones [13]. The deficiency of endogenous Mn SOD might explain the proliferative capacity of certain rare surviving cell clones [32]. In other experimental models, it was shown that, if exposure of fibroblasts to a small amount of oxygenated radicals favours their proliferation [31,32], the addition of compounds with SOD objective activity leads to the differentiation of various cell types and the arrest of proliferation [2,3]. In addition, overexpression of Mn SOD led to phenotype reversion in fibroblasts transformed by SV40 [5]. However, as most of the suffering cells are in a chronically inflammatory environment in the fibrotic tissue, the most likely explanation seems to be the exhaustion of antioxidant defenses by senescence. Thus, in our human in vitro model, the rise in Mn SOD observed after LipSOD treatment might allow the partial restoration of these cellular defenses, thus helping to normalize the fibroblast phenotype. In many studies, intracellular Mn SOD has been reported to play a specific role in relation to various cytokines. Tumor necrosis factor (TNF) α and β, interleukin 1 and interferon γ can induce a rise in Mn SOD expression in vitro and in vivo in epithelial or mesenchymal cells. In those cases, these cytokines would play a protective antioxidant role in relation to healthy cells, and a selectively cytotoxic role in relation to infected cells. Conversely, overexpression of Mn SOD may inhibit TNF-induced cytotoxicity, especially apoptosis, which varies depending on the receptivity of the different cell types [16]. In the light of these considerations, we postulated that in RIF, LipSOD might block the RIF cell death and then foster a process of phenotypic reversion, as proposed in other models [17].

In conclusion, restoration of the endogenous Mn SOD deficiency might correspond to the reconstitution of the molecules responsible for antioxidant defenses rather than to the reduction of the excessive proliferation of some hyperactive fibroblastic clones.

4.2. LipSOD modulation of TGF-β1 and regulation of matrix degradation

In vivo, early active RIF is usually characterized by the chronic activation of fibroblasts in myofibroblasts with excessive proliferation and extracellular matrix (ECM) deposition [21]. The cells of this tissue remain hyperactive and secretory for a long time [18] and are controlled by chemotactic and mitogenic growth factors [35]. In this process, TGF-β1 seems to be a key element responsible for initiating, developing and maintaining this permanent local stimulation [1,4,26]: initially, it does so by paracrine secretion during the acute inflammatory evolution, and then by autocrine secretion of the fibroblasts themselves during late activation in which TIMP inhibits the metalloproteases or collagenases [15,27,28]. Because the myofibroblasts are deprived of degradation enzymes by TIMP, they accumulate matrix macromolecules, and gradual fibrotic densification sets in [19,21,24].

In vitro, early active RIF myofibroblasts are also characterized by repression of collagenases and stimulation of TIMP expression and activities. These alterations are amplified by TGF-β1 secretion, which, while stimulating fibroblast proliferation, favours extracellular matrix synthesis and reduces procollagenase expression, while enhancing TIMP accumulation [37]. Contrarily to what we observed in vivo in human fibrous tissue [9], we did not observe any overexpression of TIMP nor TGF-β1 in RIF cells in vitro. Furthermore, recent reviews suggest that the response to TGF-β1, that depends on the cell type, is ubiquitous and heterogeneous [26]: for instance, TGF-β1 may induce excessive proliferation of the fibroblasts involved in fibrosis [25], but may also induce apoptotic processes in epithelial cells such as human hepatoma cells [23] or leukemic cells [34]. Such apoptotic process might partly explain the high cell mortality observed in vitro when cultures of human RIF fibroblasts are initiated.

In vivo, at the extracellular level, Cu/Zn SOD exerts anti-inflammatory activity but not direct enzymatic action on matrix degradation [29]. Our in vitro results support the hypothesis of an indirect effect on matrix deposition, as LipSOD reduced TIMP expression at the transcriptional level in human RIF fibroblasts. This reduction may result in an increased capacity for matrix degradation, due to the removal of collagenase inhibition. Concomitantly, we observed a reduced expression of TGF-β1. This reduction might explain the breaking of the chronic stimulation of the fibrotic process and the reduction of TIMP gene expression. Exposure to LipSOD seems to tip the balance between synthesis and degradation of the collagen matrix in favour of degradation, and might lead, not only to the arrest of RIF aggravation, but also, and above all, to the dissolution of the pre-existing matrix network.

To sum up, treatment with exogenous LipSOD seems to have an in vitro 'antifibrotic' action. This may occur by partial restoration of endogenous Mn SOD, TGF-β1 and TIMP expressions. However, because of the small amount of human tissue which was available and the small cell extraction yield, we were unable to confirm these mechanisms by exploring the proliferative behaviour of cells in culture after exposure to LipSOD. Recently, additional works in pig skin cell culture have been realized with LipSOD modulation on early active RIF myofibroblasts versus normal fibroblasts. In these pig cells, SOD have reduced significantly the overexpression of protein and

mRNA TGF-β1 in RIF myofibroblasts versus normal fibroblasts [36]. SOD have also lowered the levels of the markers αsm-actin and β-actin and of extracelluar matrix components α1(I) collagen and tenascin C, suggesting the reversion of RIF myofibroblasts into normal fibroblasts [36]. That 'antifibrotic' effect corroborated with a reduction of TGF-β1 expression was also verified recently in our pig model at the tissular level [22], confirming our clinical phase II trial [14] using a combination of pentoxifylline-tocopherol.

5. Conclusion

Our observations on radiation-induced fibrosis and its in vivo reversibility have opened up prospects for new treatment, and we are now able to modify the concept of irreversibility of chronic radiotherapy damage. The treatments designed to weaken the fibrotic process could act by modulating cell phenotype rather than by destroying pathologic scars. Although the role of SOD is the dismutation of the superoxide radical, it may also exert many other indirect biological effects which are currently being identified. We therefore postulate that in addition to its expected anti-inflammatory action, LipSOD permits the reversion of the phenotype of RIF cells by restoring the disturbed balances of cell proliferation and matrix synthesis and degradation in a process of 'programed cell normalization'.

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