STRIKING REGRESSION OF SUBCUTANEOUS FIBROSIS INDUCED BY HIGH DOSES OF GAMMA RAYS USING A COMBINATION OF PENTOXIFYLLINE AND α-TOCOPHEROL: AN EXPERIMENTAL STUDY

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Purpose: To establish a successful treatment of subcutaneous fibrosis developing after high doses of gamma rays, suitable for use in clinical practice.

Methods and Materials: We used an animal model of acute localized gamma irradiation simulating accidental overexposure in humans. Three groups of 5 Large White pigs were irradiated using a collimated 192Ir source to deliver a single dose of 160 Gy onto the skin surface (100%) of the outer side of the thigh. A well-defined block of necrosis developed within a few weeks which had healed after 26 weeks to leave a block of subcutaneous fibrosis involving skin and skeletal muscle. One experimental group of 5 pigs was dosed orally for 26 weeks starting 26 weeks after irradiation with 1600 mg/120 kg body weight of pentoxifylline (PTX) included in the reconstituted food during its fabrication, and another group of 5 was dosed orally for the same period with a daily dose of 1600 mg/120 kg body weight of PTX combined with 2000 IU/120 kg body weight of α-tocopherol. Five irradiated control pigs were given normal food only. Animals were assessed for changes in the density of the palpated fibrotic block and in the dimensions of the projected cutaneous surface. Depth of scar tissue was determined by ultrasound. Physical and sonographic findings were confirmed by autopsy 26 weeks after treatment started. The density, length, width, and depth of the block of fibrotic scar tissue, and the areas and volume of its projected cutaneous surface, were compared before treatment, 6 and 13 weeks thereafter, and at 26 weeks.

Results: The experimental animals exhibited no change in behavior and no abnormal clinical or anatomic signs. No modifications were observed in the block of fibrotic scar tissue of pigs dosed with PTX alone. However, significant softening and shrinking of this block were noted in the pigs dosed with PTX + α-tocopherol 13 weeks after treatment started and at autopsy, when mean regression was ~30% for length, ~50% for width and depth, and ~70% for area and volume. Histologic examination showed completely normal muscle and subcutaneous tissue surrounding the residual scar tissue. The 50% decrease in the linear dimensions of the scar tissue, were comparable to the results obtained in our previous clinical studies, and were highly significant compared to the clinical and autopsy results for the controls. Histologic examination of the residual scar tissue revealed tissue which was more homogenous and less cellular and inflammatory than in control and PTX-dosed pigs. The histologic examination showed complete normal muscle and subcutaneous tissue surrounding the residual scar tissue. The 50% decrease in the linear dimensions of the scar tissue, were comparable to the results obtained in our previous clinical studies, and were highly significant compared to the clinical and autopsy results for the controls. Histologic examination of the residual scar tissue revealed tissue which was more homogenous and less cellular and inflammatory than in control and PTX-dosed pigs. The histologic examination showed complete normal muscle and subcutaneous tissue surrounding the residual scar tissue.

Conclusions: The present results showed a striking regression of the subcutaneous fibrotic scar tissue that develops as a consequence of high doses of gamma rays. © 1999 Elsevier Science Inc.

Pentoxifylline, alpha-tocopherol (α-tocopherol), Late radiation effects, Radiation-induced fibrosis, Tumor necrosis factor alpha (TNFα), Transforming growth factor beta-1 (TGFβ-1).

INTRODUCTION

The potential efficacy of radiation therapy for malignant tumors is limited by the need to avoid late damage to normal tissue. Although the development of new strategies to improve the therapeutic success rate has reduced the incidence of severe radiation-induced fibrosis (RIF), radiation therapy sequelae are still sometimes unavoidable, and can cause great handicaps in a significant number of patients (1–4). RIF is notoriously difficult to manage and does not regress.

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spontaneously. Thus, in the management of 34 patients presenting with 42 RIF lesions with a mean regression of about 60% in area, only superoxide dismutase (SOD), as bovine liposomal Cu/Zn-SOD, proved clinically useful (5).

To study the fibrotic process that follows accidental radiation overexposure and to develop effective new therapies, our laboratory, working as part of a European Radiation-protection Experimental Network, has developed a standardized pig model simulating accidental overexposure to γ radiation (6). Localized accidental overexposure induces severe acute tissue damage that progresses through erythema to necrosis at the site of contact. Depending on the radiation dose received, the necrosis may heal, leaving a various extensive fibrotic block (FB) involving skin and skeletal muscle (7). In this model, members of our team have studied the macro- and microscopic anatomy of the FB (8), its biochemistry (9, 10), and its behavior in cell culture (11–13). The results of these studies suggested that in FB the expression of the cell phenotypes in vivo and in vitro are greatly altered and that transforming growth factor beta-1 (TGF-β1) played important roles in promoting and regulating the late fibrotic process (14, 15). In this experimental pig model of cutaneous muscular FB, we showed that, whether the treated animals were given Cu/Zn-SOD or Mn-SOD, all of them underwent significant and roughly equivalent softening and shrinking of the fibrotic scar tissue, when mean regression was about 30% for depth, and about 70% for area and volume (16). These results were comparable to our earlier clinical findings (5). Today, however, this treatment is no longer available, and that is why we tried to develop an alternative treatment with pentoxifylline (PTX), either alone or combined with another antioxidant such as α-tocopherol.

PTX, a methylxanthine derivative, was initially characterized as an agent which enhances red blood cell deformability and reduces blood viscosity by inhibiting platelet aggregation. It has therefore been used for the symptomatic treatment of various vascular disorders, including intermittent claudication, ischemic leg ulcers, and peripheral vascular diseases. Favorable results have also been reported for PTX in several skin disorders, suggesting that it may also have significant anti-inflammatory effects. Most studies to date have focused on the effect of PTX on the production and function of tumor necrosis factor alpha (TNFα) (17).

During the last 10 years, different experimental and clinical trials showed that PTX had a potential effect against injuries to normal tissue caused by radiation. Dion et al. (18) initially described a reduced incidence of severe skin and soft tissue toxicity after radiation of the extremities, in mice treated with PTX. These investigators subsequently reported that 400–800 mg/day of PTX appeared to enhance the healing of chronic radiation-induced mucocutaneous ulcers in 12 patients (19). All these patients had pain relief, and this relief was also mentioned in a case report, published in 1993, concerning painful postradiation fibrosis (20). More recently, in 1997, similar observations were reported by Futran et al. (21) in 26 patients with soft tissue necrosis or mucosal injuries following radiotherapy for head and neck cancer, who had been treated with 1200 mg/day of PTX. Lastly, when administered for several months in a dose range of 400–1200 mg/day, PTX reduced pain and accelerated the healing of radiation-induced necrosis of the soft tissues, and may also have had a beneficial antifibrotic effect. In addition, an uncontrolled clinical trial with α-tocopherol (average dose: 700 mg/day for 3.5 months) involving 53 patients with RIF induced by brachytherapy for breast cancer showed a mean reduction of about 20% in the average diameter of the fibrotic lesions (22).

All these results prompted us to start a preliminary study with a combination of PTX and α-tocopherol for the treatment of superficial RIF. This study included 10 patients without radiation necrosis but with a palpable zone of superficial RIF involving the skin and subcutaneous tissues. These fibrotic lesions were induced by conventional radiation therapy for head and neck, and breast cancer 5.5 ± 3 years previously, using a mean total dose of 66 ± 15 Gy. Eighty percent of the patients presented with functional sequelae such as restricted movements, edema, and/or signs of inflammation. PTX (800 mg/day) and α-tocopherol (1000 IU/day) were administered orally for 6 months. Treatment was well tolerated and all the patients exhibited clinical regression of the RIF and functional improvement. Clinically assessable regression began during the second month of treatment, and at 6 months, the maximum linear dimension and area of the projected cutaneous surface of the RIF had regressed by 45 and 64% respectively (23). During the same period, an empirical case report showed that the combination of 1200 mg/day PTX and 400 mg/day vitamin E reduced the thickness of dermis, as visualized by 20 MHz ultrasound scans, in a skin field treated by radiation therapy 17 years previously (24).

Because the subcutaneous fibrotic sequelae of radiation exhibit the same basic histologic characteristics, regardless of differences in the etiopathologic factors, we decided to use our experimental pig model of localized overexposure to radiation for further study and comparison of the effects on FB of PTX, either alone or combined with α-tocopherol, under the same strictly controlled experimental conditions as those used for our previous experimental SOD trials (16). The present report gives the results of this comparative study conducted in 15 experimental pigs with well-characterized zones of fibrotic scar tissue in the skin and skeletal muscle of the outer side of the thigh.

**METHODS AND MATERIALS**

The study was performed in the Laboratoire de Radiobiologie et d’Etude du Génome, using castrated Large White male pigs weighing 45 ± 5 kg (Permits no. 3255 and 5564 of the Animal Protection Office of the French Ministry of Agriculture and Forestry).

**Induction of fibrosis**

Fifteen animals were irradiated under anesthesia (66% O₂ + 33% NO₂ and 2% halothane) on the outer side of the
thigh with a collimated source of $^{192}$Ir (skin-source distance: 1.7 cm; dose rate: 8 Gy/min; $E_{m}(\gamma) = 0.38$ MeV) (25). A single dose of 160 Gy was delivered to the skin surface (100%) corresponding to a dose of 40 Gy at a depth of 2 cm (25%). The irradiated field, defined as the area lying within the 20 Gy isodose (12.5%), corresponded to a trapezoid-shaped zone delimited by the formation of an area of moist desquamation, 7 cm long and 3 to 6 cm wide. A well-defined volume of necrosis developed within a few weeks which had healed after 26 weeks to leave a block of subcutaneous fibrosis involving skin and skeletal muscle (6, 7).

**Assessment of the fibrotic zones before treatment**

Twenty-six weeks after irradiation, pigs were examined under anesthesia (66% O$_2$ + 33% NO$_2$ and 2% halothane). Palpation of the irradiated thigh revealed a well-defined block of dense hard fibrosis involving the skin, subcutaneous adipose tissue, and biceps femoris muscle. The limits of the projected cutaneous surface of this fibrotic block were outlined on tracing paper and computerized, and its surface area, length, and maximum width were measured. The depth of the FB was determined by ultrasound (Scanner 200 Pie Medical, Hospimédi, France) with a 5 MHz linear electronic probe. In this experimental series of 15 pigs, the mean dimensions of the block were as follows: length: 9.4 ± 1.4 cm; width: 7.3 ± 1.0 cm; depth: 3.6 ± 0.3 cm; surface area: 47.3 ± 9.9 cm$^2$; and volume: 107 ± 13 cm$^3$ (hemispheric volume $= \frac{2}{3} \times \text{area} \times \text{depth}$). This volume was larger than that of the zone given a high dose of irradiation (16).

**Treatment**

Twenty-six weeks after irradiation, the mean body weight of the 15 pigs was 112 ± 19 kg. Each one was housed individually and given 2 meals/day of reconstituted food. PTX alone (Hoechst Laboratories, Paris, France), or the combination PTX + $\alpha$-tocopherol (DL $\alpha$-tocopherol acetate, MicrovitTM Promix 50, France) was incorporated into the reconstituted food during its fabrication by the manufacturer. Dosages and administration modalities were based on those used in our previous clinical trials (23, 26). Accordingly, 1600 mg/120 kg body weight of PTX was administered orally to 5 pigs every day for 26 weeks, and 1600 mg PTX plus 2000 IU $\alpha$-tocopherol, to another 5 pigs for the same period. The remaining 5 were controls, which were given normal food without the above supplements.

**Evaluation of residual fibrotic zones**

Pigs were examined under anesthesia 6, 13, and 26 weeks after the beginning of the above treatment. They were killed under deep anesthesia at the end of treatment, for comparison of clinical and sonographic findings, and to provide samples for histopathological studies. The length, width, and depth of the residual fibrotic block, the area of its projected cutaneous surface, and its calculated volume before treatment were compared to the corresponding measurements 6, 13, and 26 weeks after the treatment started and expressed as the regression, in percent, of the pretreatment measurements. Data were analyzed using Student’s $t$-test. Changes in the density of the fibrotic block were noted and scored as ± for slight but perceptible softening and ++ for significant softening. The +++ score for very pronounced softening indicated that the original zone of fibrosis could barely be distinguished from the surrounding tissue.

**Histology and indirect immunofluorescence of TNF$\alpha$ and TGF$\beta$**

Tissue samples were fixed in 10% formalin-buffered solution and embedded in paraffin wax. Serial 5-μm sections were cut, dewaxed, and either colored with hematoxylin–eosin–safranin or permeabilized with 0.1% triton solution for immunofluorescence labeling. After permeabilization, sections were washed in phosphate-buffered solution (PBS), saturated with 2% bovine serum albumin (BSA) solution in PBS, and incubated overnight at 4°C with the primary antibody. They were then washed 3 times in PBS, incubated for 45 min with the secondary antibody, mounted in PBS–glycerol (v/v), and examined with a BH2 Olympus epifluorescence microscope. Each antibody was tested in order to obtain the concentration that gave maximal specific and minimal background fluorescence. The primary anti-TNF$\alpha$ antibody, a polyclonal antibody directed against purified recombinant human TNF$\alpha$ (Genzyme Diagnostics), was used at dilutions ranging from 1/100 to 1/250. A 1/150 dilution was used for magnification. The primary anti-TGF$\beta$-1 antibody, a polyclonal antibody directed against a peptide corresponding to amino acids 328–353 of the carboxy terminal region of human TGF$\beta$-1 (Santa Cruz Biotechnology), was used at dilutions ranging from 1/50 to 1/150. A 1/50 dilution was used for magnification. The second fluorescein isothiocyanate (FITC)–rabbit antibody (Immunotech) was used at a dilution of 1/100.

**RESULTS**

**Quantitative changes**

Control pigs displayed fibrotic zones of various sizes, with standard deviations ranging from ±0.2 cm for depth to ±1.8 cm for the length. However, no changes in fibrotic zone size were observed as a function of time. The maximum variations, ranging from −10% to +3%, corresponded to the methodological limits of precision in this model.

Compared to the control pigs, fibrosis did not regress in those dosed with PTX alone at any of the periods evaluated; it did regress significantly in pigs dosed with PTX + $\alpha$-tocopherol as measured 6, 13, and 26 weeks after the dosing started (Table 1).

At 6 weeks, mean fibrotic zone length, width, and depth had regressed by 6, 14, and 12% respectively in pigs treated with PTX + $\alpha$-tocopherol but the differences compared to the predosing measurements were not significant. At 13 weeks, length, width, and depth had regressed significantly by 21, 31, and 35%, respectively. At autopsy, i.e. at 26...
Table 1. Mean % regression of the fibrotic block after 6, 13, and 26 weeks of treatment with PTX and PTX–α-tocopherol in pigs.

<table>
<thead>
<tr>
<th>Time from start of dosing</th>
<th>Control pigs</th>
<th>Pigs dosed with PTX</th>
<th>Pigs dosed with PTX+α-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks</td>
<td>L 1 ± 4%</td>
<td>0 ± 4%</td>
<td>−6 ± 9%</td>
</tr>
<tr>
<td></td>
<td>W −4 ± 3%</td>
<td>1 ± 6%</td>
<td>−14 ± 7%*</td>
</tr>
<tr>
<td></td>
<td>D 0 ± 6%</td>
<td>2 ± 4%</td>
<td>−12 ± 8%</td>
</tr>
<tr>
<td>13 weeks</td>
<td>L 3 ± 5%</td>
<td>2 ± 8%</td>
<td>−21 ± 10%*</td>
</tr>
<tr>
<td></td>
<td>W −10 ± 8%</td>
<td>−4 ± 9%</td>
<td>−31 ± 6%†</td>
</tr>
<tr>
<td></td>
<td>D −5 ± 11%</td>
<td>1 ± 10%</td>
<td>−35 ± 16%†</td>
</tr>
<tr>
<td>26 weeks</td>
<td>L 1 ± 10%</td>
<td>3 ± 7%</td>
<td>−28 ± 7%*</td>
</tr>
<tr>
<td></td>
<td>W −7 ± 2%</td>
<td>−6 ± 10%</td>
<td>−48 ± 7%‡</td>
</tr>
<tr>
<td></td>
<td>D 2 ± 10%</td>
<td>−2 ± 10%</td>
<td>−52 ± 7%‡</td>
</tr>
</tbody>
</table>

Abbreviations: PTX = pentoxifylline; L and W = linear length and width; D = depth measured by ultrasound scans.

Means comparison of the linear dimensions, Student t-test: *p < 0.05; †p < 0.01; ‡p < 0.001.

weeks, length, width, and depth had regressed even more significantly, by 28, 48, and 52%, respectively. With regard to depth, there was no difference between the measurements made at autopsy and the sonographic evaluations (Figs. 1a and 1b).

Compared to control pigs, the measured area of the projected cutaneous area of the fibrotic block (A) and the calculated volume (V) were equivalent in the PTX-dosed pigs at each time evaluated, but were significantly smaller in the PTX + α-tocopherol–dosed pigs at 13 and 26 weeks (Fig. 2).

 Chronology and qualitative changes

Various degrees of clinically assessable regression began to appear in PTX + α-tocopherol–dosed pigs at the second evaluation, 13 weeks after treatment started, and significant and roughly equivalent regression was obtained in all 5 treated animals. Thereafter, the regression of fibrous tissue continued at an equivalent rate until autopsy at 26 weeks.

Fibrotic attachment of the skin to subcutaneous muscle tissue did not regress significantly in the control or PTX-dosed animals. However, in the PTX + α-tocopherol–dosed group, the skin could be moved freely over all the residual fibrotic blocks by the third month. The shrinking fibrotic zones became gradually softened. At autopsy, the 5 control pigs and the 5 PTX-dosed pigs exhibited hard scar tissue, scored ± for residual fibrosis softening. However, 2 of the 5 PTX + α-tocopherol–dosed pigs were scored ++ for residual fibrosis softening, and 3 were scored +++.

 Tolerance

Neither of the two drug-dosed groups of pigs displayed any significant changes in behavior during treatment.

 Macroscopic and microscopic observations

Changes in the three-dimensional form of the fibrotic block and in the histologic structure of the RIF tissue were studied at autopsy. As usually occurs in this experimental model, the control animals displayed hemispheric fibrotic zones of a “standard size,” with irregular borders infiltrating into the adjacent skeletal muscle, thus forming larger total volumes of fibrotic tissue than the volume that had received a high dose of irradiation. Histologic examination revealed the usual heterogeneous fibrosis, with central zones comprising dense long regular cords of collagen fibers, widely scattered fibroblasts, inflammatory white cells, and non-functional atrophic vascularization. Towards the periphery of this dense sclerotic tissue, which infiltrated into the surrounding muscle, zones of loose intricate fibrous stroma were observed, containing numerous newly formed vascular channels, myofibroblasts, inflammatory white cells, and atrophic muscle fibers. No significant differences were noted between fibrotic scar tissues of control and PTX-dosed pigs.

In all the PTX + α-tocopherol–dosed pigs, the residual fibrosis formed a soft sharply outlined funnel-shaped cord as the volume of fibrotic tissue decreased. This change in form made it difficult to compare tissue volumes before and after treatment, because the calculated volumes referred to a hemisphere, and overestimated the volume of the funnel-shaped cord. Histologic examination of the residual fibrosis revealed a more homogeneous tissue, which was less cellular and inflammatory than that of the PTX-dosed and untreated control pigs.

 TNFα and TGFβ-1 immunohistological observations

The cellular sources of TNFα and TGFβ-1 proteins, and their levels of expression, were examined in paraffin sections by immunofluorescence.

In the control pigs, strong TNFα expression was detected in the normal skin: thus, a decreasing labeling gradient was observed in the keratinocytes of the epidermis, from the stratum corneum to the basal layer of the epidermis. In the dermis, labeling was observed in the cytoplasm of the different cell types (fibroblasts, endothelial cells, and muscular fibers) as well as in the extracellular matrix. In the fibrotic skin, anti-TNFα labeling was stronger in the extracellular matrix, and particularly in the myofibroblasts, but its tissue distribution was not significantly different from that seen in the normal skin.

Neither PTX nor PTX + α-tocopherol induced significant differences in the level of TNFα expression in the cellular or extracellular matrix localization.

In the control pigs, homogeneous TGFβ-1 expression was detected in the epidermis of the normal skin. Slight expression was detected in the fibroblasts and endothelial cell cytoplasm, but the connective matrix of the dermis was not labeled. In the fibrotic skin, anti-TGFβ-1 labeling was particularly strong in the myofibroblasts and endothelial cells and the surrounding matrix. Furthermore, strong TGFβ-1 expression was observed in the inflammatory cells and atrophic muscular fibers around the fibrotic tissue, whereas normal muscular fibers were not labeled.

In the PTX-dosed pigs, the level of expression and cellular distribution of TGFβ-1 were comparable to those of...
the control animals (Fig. 3). However, in pigs treated with PTX + α-tocopherol, an overall decrease of TGFβ-1 labeling was observed in the extracellular matrix. A slight anti-TGFβ-1 labeling was detected in the fibroblasts, endothelial cells, or atrophic muscular fibers (Fig. 4), but the few remaining inflammatory cells were still labeled with the anti-TGFβ-1 antibody.

**DISCUSSION**

Our previous clinical findings challenged the postulate that RIF is irreversible, as they demonstrated that systemic treatment by SOD significantly reduced long-standing fibrosis following radiation therapy (5). Further, our experimental results were comparable to the clinical findings in this respect (16). However, as stated in the Introduction, this treatment is no longer available, which is why we tried to develop an alternative treatment with PTX, either alone or combined with α-tocopherol.

In the present study, we observed that in the pigs treated with PTX + α-tocopherol, the volume and area of the fibrosis that develops as a consequence of radiation-induced necrosis, regressed by a mean of about 70%. This is comparable to the fibrotic block regression previously observed in SOD-treated pigs. However, the periods over which it occurred were different as our present results were obtained 6 months after the treatment started, whereas in the SOD-treated pigs the same results were obtained after only 2 months (16).

**Pentoxifylline**

PTX has been used therapeutically for a long time in very large numbers of patients suffering from vascular disorders. It has been reported to exert strong effects on platelets, endothelial cells, leukocytes, and macrophages. PTX increases the phagocytic activity of polymorphonuclear leukocytes (PMN) and monocytes, antagonizes TNFα production and activity by murine and human leukocytes and granulocytes, and reduces in vitro production of multiple cytokines, including granulocyte-macrophage colony stimulating factor (GM-CSF) and gamma-interferon (IFNg).

In the pig, PTX has been shown to enhance the healing of full-thickness skin flaps in normal skin (27) and to have an antifibrotic effect in liver sclerosis induced by yellow phosphorus (28). In the rat, this antifibrotic effect was not observed in the liver fibrosis induced by bile duct ligation (29, 30) but was observed in bleomycin-induced alveolitis (31).

In radiobiology, PTX displayed a radioprotective effect in various studies: in vitro, a preincubation with PTX of the HL-60 cell line inhibited both the expression of the radiation-inducible genes c-jun and Egr-1, and the increase of the protein kinase C (32); in vivo, pretreatment of mice with PTX before irradiation suppressed the radiation-induced gene expression of interleukins (IL), IFNg, TNFα and β, and intercellular adhesion molecules (ICAM-1) in the brain (33). However, when administered in vivo before or after irradiation, PTX had either a transient beneficial effect or none, depending on the dose, on acute reactions to radiation in rat lung and skin (34–37), and no effect on the incidence or latency of late rectal ulcer healing in rats (38). However, PTX did have a moderate protective effect on normal tissues, by reducing late reactions in the subcutaneous connective tissue of mice (18) and in the lung of rats (34).

In the present study, in which pigs were treated 26 weeks after irradiation, we did not observe changes in the fibrotic block of those treated with PTX only, as suspected from the clinical reports by Dion et al. (19) and Futran et al. (21), although the doses of PTX administered and the duration of administration were very similar.

In vitro studies indicated that PTX might be a potentially antifibrotic agent. In cultured normal human keratinocytes...
and Langerhans cells, PTX modulated ICAM-1 expression (39). In cultured normal human dermal fibroblasts, it inhibited the proliferation driven by serum and IL1-β, and the production of collagen, glycosaminoglycan (GAG), and fibronectin, and stimulated collagenase activity (40). In fibroblasts derived from different abnormal human fibrotic skin, PTX inhibited the proliferation and production of GAG and fibronectin, but had no effect on collagenase activity (41–43). In normal fibroblasts, it has been shown to block the induction of collagen by TNFα and GAG synthesis, at a locus different from that of the TNFα receptors (44). PTX has also been shown to lower the levels of type I and III procollagen mRNA, and of nuclear factor 1 (NF-1), a procollagen gene-activating transcription factor, in human dermal fibroblasts (45).

Lastly, although these in vitro studies indicated that PTX might be a potentially antifibrotic agent, the high concentrations necessary to suppress fibroblast collagen synthesis suggested that routine oral doses of PTX would not be effective as an antifibrotic therapy (40, 41). Furthermore, in the in vivo experimental models, PTX, used alone before or during the experimental injury, finally displayed a relatively poor antifibrotic effect, depending on the dose administered, and on the time or duration of administration, which may differ among species and under different experimental conditions (29).

α-Tocopherol

Fifty years ago, it was observed that deficiency of vitamin E (i.e., α-tocopherol) was associated with abnormal repair

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**Fig. 2.** Mean regression of the projected cutaneous surface of the residual fibrotic block (Area) and the calculated hemispheric volume (Volume) measured in irradiated pig thigh after 6, 13, and 26 weeks of treatment with either PTX alone or PTX + α-tocopherol. (PTX = pentoxifylline). (Means comparison, Student’s t-test: *p < 0.05; **p < 0.01; ***p < 0.001).
of connective tissue, which resulted in the production of human scar-like tissue. Today, the antioxidant role of vitamin E in biological systems is well known. Catalase, SOD, and glutathione peroxidase are the main enzymes implicated in the antioxidant defenses, and the involvement of SOD in the prevention of radiation damage is well documented. Vitamins C and E are the main constituents of the nonenzymatic defenses, and both are also considered to be radioprotectors. Reactive oxygen species (ROS), such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals, are generated during inflammatory reactions and RIF development. When ROS are not scavenged efficiently, oxidative stress may result and lead to cell necrosis or apoptosis.

α-Tocopherol is located primarily in cellular membranes and is the most important antioxidant that protects membrane phospholipids from oxidative damage. Apart from this protection, it has been reported to have other functions, such as the maintenance of energy metabolism and protection of membrane proteins. These proteins are intracellular macromolecules liable to be attacked by ROS, which may cause irreversible damage.

Experimental and clinical evidence indicates that the development of fibrosis in the lung, kidney, and liver is generally associated with the overexpression of TGFβ-1, increased transcription of procollagen type I, and lipid peroxidation of biological membranes, as shown by malondialdehyde (MDA) production. In bleomycin-induced lung fibrosis models, in which ROS are highly induced by this antineoplastic drug, α-tocopherol, administrated before or concomitantly with the drug, considerably reduced the fibrotic effect of bleomycin on lung tissues of mice and rats (46, 47). Comparison of the effects of several antioxidants in a mouse model of lung fibrosis showed that only α-tocopherol, SOD, and copper biomimetic SOD significantly reduced fibrosis development in the lung tissue (48). In a model of glomerular and tubulointerstitial injury, oral supplementation of α-tocopherol inhibited the concentrations of TGFβ-1 mRNA and plasma MDA (49). In a carbon tetrachloride–induced liver fibrosis rat model, long-term oral α-tocopherol supplementation inhibited the levels of liver TGFβ-1 and procollagen mRNA. Moreover, this supplementation also downregulated basal levels of TGFβ-1 mRNA in the liver of the control nonfibrotic rats (50). In cultured human hepatic stellate cells, the major source of collagen in the liver, the increase in procollagen I mRNA expression and synthesis induced by exposure to neutrophil-derived ROS, was also inhibited by the addition of antioxidants such as α-tocopherol or SOD (51).

Finally, these examples of in vivo and in vitro studies indicate that the antioxidant α-tocopherol might be a potential antifibrotic molecule because it inhibited the overexpression of TGFβ-1 and the transcription of type I collagen. However, as observed in the treatment of Peyronie’s disease (52), the clinical use of α-tocopherol alone in patients suffering from RIF only had a moderately beneficial antifibrotic effect (22) as the mean regression of the fibrotic lesions was only about 20%. Furthermore, half the cases of RIF which diminished or disappeared during α-tocopherol treatment recurred at the end of this treatment. In the trial concerned, the duration of administration (3–4 months) was probably not long enough to obtain a stable significant antifibrotic effect.

In the present study, we observed a mean regression of about 70% of the volume of the fibrotic block in the pigs treated with PTX + α-tocopherol combination. This regression may have been due to a synergistic effect of these two molecules on extracellular matrix regulation. This hypothesis is supported by the present results for TNFα and TGFβ-1 immunolocalization observed in the residual fibrotic tissues of the pigs treated with PTX + α-tocopherol.

In vivo cell death or myofibroblast phenotypic reversion?

TGFβ-1 is considered as a key cytokine whose sustained synthesis underlies the development of tissue fibrosis. It is
strongly chemotactic for fibroblasts and induces them to secrete extracellular matrix proteins. TGFβ-1 exhibits auto-induction potency and modulates the action of platelet-derived growth factor, fibroblast growth factor, IL-1, and TNFα in such a way that it can probably be considered to “orchestrate” tissue fibrosis (53). After radiation injury, the release of cytokines and growth factors in irradiated tissues perpetuates and augments the inflammatory response, while promoting fibroblast recruitment and proliferation (14, 15). An amplified response to injury by both the endothelial and connective tissue cells, possibly due to persistent modifications in the genetic programming of differentiation and proliferation (54), leads to the histologic modifications that characterize RIF. Large numbers of myofibroblasts, which are cells that have acquired a contractile phenotype, persist for long periods in long-standing fibrotic tissue and then progressively disappear, leaving only a sparse cell population. The almost complete regression of fibrosis noted here in the pigs treated with PTX + α-tocopherol, as well as the regression we previously observed in SOD-treated animals (16), suggest that great changes take place in the genetic programming of the cell differentiation and proliferation that characterize RIF (54). This regression implies the death of the cells which constituted the fibrotic tissue before the treatment. In addition, the complex interactions between certain cytokines and intracellular Mn-SOD that affect the genetic expression of cells (55, 56) seem to indicate that in RIF, treatment with antioxidants, i.e., α-tocopherol or SOD, might lead to in vivo phenotype reversion of myofibroblasts and endothelial cells. This might occur, either because such treatment modifies cell responses to growth factors and other cytokines, or because it exerts a direct effect on genetic programming. Both mechanisms of cell death and phenotypic reversion are currently investigated in our in vitro model (57) of RIF.

CONCLUSION

To our knowledge, this is the first time that PTX combined with α-tocopherol has been shown to have a significant antifibrotic effect in vivo, reversing the fibrosis that develops as a consequence of high doses of gamma rays. These results are comparable to those obtained in our preliminary clinical studies. The 70% decrease in the volume of the fibrotic block is highly significant compared to the results for pigs treated with PTX alone and for control animals. This demonstration raises many questions, mainly about the precise mechanisms of action in vivo of the PTX-α-tocopherol combination, and it is necessary to await the results of ongoing cellular and molecular studies before these mechanisms can be identified.

REFERENCES

21. Futran ND, Trotti A, Gwede C. Pentoxifylline in the treatment