Effects of superoxide dismutase topical treatment on human skin radiofibrosis: a pathological study

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SUMMARY. Fourty-two patients, presenting with clinically evaluable cutaneous fibrosis after radiotherapy for breast carcinoma, were treated for 3 months by a superoxide dismutase (SOD) topical preparation. Sequential cutaneous punch biopsies were performed before and 3 months after completion of the treatment. The histochemical grading, using an objective spectrometric method, showed a decrease of fibrosis in 74% of treated patients. The immunohistochemical expression of smooth muscle cells (SMC) α-actin, epidermal growth factor receptor (EGFR) and transforming growth factor β1 (TGFβ1) was studied. SOD treatment resulted in an increase of SMC α-actin expressing fibroblasts in 79% and an increase of EGFR expressing epidermal cells in 67% cases. It did not modify the expression of TGFβ1. In conclusion, topical SOD appeared to reduce collagen accumulation in the dermis of irradiated skin and to ‘reactivate’ cellular functions in dermal fibroblasts and epidermal cells, as demonstrated by phenotypic changes.

INTRODUCTION

Radiation-induced fibrosis represents a late complication of exposure to ionizing radiation. Its incidence varies from 3% to 20%1-3 of breast cancer patients treated by breast-conserving treatment with irradiation. The radiosensitivity of the connective tissue and induction of fibrogenesis vary from one patient to another. In the irradiated skin, the histopathological changes are characterized by an early sterilization of the epidermal basal layer cells, followed by an epidermal cellular depletion and a dermal ‘atrophy’, consisting in a decrease in the number of fibroblasts and an increase of collagen deposition.4

The occurrence of radiofibrosis increases with time from treatment. Free radicals, i.e. superoxide (O₂⁻) and hydroxyl (OH·), produced by radiolysis at the time of irradiation5-6 may induce molecular lesions in endothelial cells. Early reactions are inflammatory, ischaemic, and occasionally necrosis and ulceration are seen.7 Later, fibrosis is progressively established and may generate pain and motor disability, when the axilla is involved. Therapeutic approaches using ‘radical scavengers’, like superoxide dismutase (SOD), were initiated some years ago.8,9 SOD refers to a family of metalloprotein enzymes, that have radioprotective and anti-inflammatory actions,10 but its ‘antifibrotic’ role is debated. No toxicity has been observed, when proper purification of the protein was obtained.12

The clinical effects of this enzyme in radiation-induced acute injuries have been studied in our Institute for many years. Therapeutic trials have been performed on radiofibrosis13-14 at various sites (skin, rectal mucosa, lung). Most of them showed beneficial effects of SOD in reverting fibrosis.

The present study was initiated in 1992 and included 55 patients previously irradiated for conservative breast cancer treatment, who exhibited obvious clinical cutaneous fibrosis. We have analysed the histochemical changes in skin samples obtained from patients before and 3 months after completion of topical SOD treatment. We report here the effects of this treatment on histopathological indicators of...
skin fibrosis, including histochemical (Sirius Red staining) quantitative measurement by dye elution and spectrometry. We also report on phenotypic changes observed after SOD treatment, which include smooth muscle cells-α actin (SMC-α actin) expression, transforming growth factor β1 (TGFβ1) intracellular localization in skin cells and epidermal growth factor-receptors (EGFR) in the epidermal cells.

**MATERIALS AND METHODS**

**Patients**

From February 1992 to January 1993, 55 patients, presenting with clinically evaluable fibrosis after first line radiotherapy for breast carcinoma, received topical treatment by SOD. Thirteen of the 55 patients were excluded from the study either because they refused sequential biopsies (10 out of 13 patients), because the first biopsy was inadequate (1 patient), or there was evidence of cancer recurrence (2 patients).

The 42 evaluable patients were aged between 45 and 79 years (mean age ± SD: 58.8 ± 8.7 years). The time elapsed between irradiation and treatment by SOD varied from 3 months to 40 years (mean delay ± SD: 8.5 ± 8.4 years).

These patients were treated by Co on the breast and lymphatic areas, according to the breast conserving technique of the Institut Curie\textsuperscript{15,16} at doses ranging from 55 to 85 Gy to the tumour (mean total dose ± SD: 72.6 ± 9.8 Gy). In 19 cases, a mean dose of 20 Gy was given as localized boost. The dose delivered to the skin ranged from 5 1.7 to 77.4 Gy (mean skin dose ± SD: 60.6 ± 9.2 Gy). All patients gave informed consent for SOD treatment and serial skin biopsies.

**SOD treatment**

Non-pyrogenic SOD\textsuperscript{17} derived from bovine erythrocytes (Biogenzia–Lemania, Switzerland) and controlled for its biological activity (3650 U/mg) was used in an ointment form, prepared with polyethylene glycol (PEG) MW 3350 (Sigma Chimie, USA) as excipient and benzylic alcohol (Sigma Chimie, USA) as preservative.

The cream, packaged in pots containing 10 mg PEG-SOD (3.6 × 10⁴ U) each, was applied locally twice a day, every 12 h for 90 days. The daily dose per patient was at least 1600 U and the total dose was 40 mg (144 000 U) of SOD.

**Histological and immunohistological study**

**Samples**

Punch-biopsies 2 mm in diameter were taken from areas of identical skin doses, in patients before (M0) and 3 months after the treatment completion (M6) and then immediately frozen in liquid nitrogen. Serial cryosections 6 mm thick were obtained, pre- and post-treatment biopsies were paired. (two sections on a single slide) and were stained immediately with haematoxylin and eosin for histologic examination; other slides were stored at −20°C.

**Fibrosis grade**

For each case and each sampling time (M0, M6), two slides with four consecutive cryosections were fixed in 10% formalin, stained for 1 h with 0.1% Sirius Red in saturated picric acid\textsuperscript{18} and rinsed in 0.01 N HCl.

Two tissue sections were carefully scratched out from each glass slide and put into two glass tubes containing 1 ml of 0.1 N NaOH each. Sirius Red was thus extracted for 30 min at 37°C. The optical density (OD) was read at 540 nm in a spectrophotometer (Uvikon 810, Roche). The results were expressed in OD/cm² of cryosection surface area (measured by microplanimetry).

Two other sections on each slide were counterstained with Harris' haematoxylin. Histological grading of fibrosis was based on an arbitrary scale of five grades from 0 (normal dermis) to 4 (severe fibrosis), taking into account the relative amount of dermal cells and collagen bundles respectively. This subjective score was corrected by the OD values after elution.

**Immunohistochemistry**

The phenotypic changes of dermal fibroblasts were studied, using antihuman vimentin, 5 β-hydroxylase and SMC-α actin monoclonal antibodies (Dakopatts, Denmark). We used a monoclonal antibody MoAb 425 (Oncogene Science, USA) to reveal EGFR on epidermal cells and a polyclonal antibody anti-TGFβ1 (R & D Systems, Inc., USA), to detect this cytokine.

An indirect immunoperoxidase technique was applied as previously described.\textsuperscript{18} Briefly, after fixation with acetone at −20°C, sections were immersed in methanol-3% H₂O₂ to block endogenous peroxidases, treated for 30 min at normal non-immune serum at room temperature then incubated overnight at 0°C with the specific primary antibody. For TGFβ1 immunostaining, acidic conditions (pH 5.5) and a pre-treatment by hyaluronidase (1 mg/ml, 30 mn at 37°C) were applied. The primary antibody was detected by an avidin–biotin complex (Vectastain Biosys, USA) and a reaction with 0.7% H₂O₂ in 0.06% diaminobenzidine (Sigma Chemical Co, USA). The slides were counterstained with Harris' haematoxylin.

An arbitrary scale of five grades from 0 (normal skin) to 4, including the amount of stained cells and the intensity of labelling, was used to classify the immunohistochemical changes, as described in the Table.
Superoxide dismutase treatment of skin radiofibrosis

Table Immunohistochemical grading of SMC α-actin, EGFR and TGFβ1, based on the proportion of stained cells and the intensity of labelling

<table>
<thead>
<tr>
<th>Grades</th>
<th>SMC-α actin</th>
<th>EGFR</th>
<th>TGFβ1</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Normal distribution (dermal vessels)</td>
<td>Normal distribution (basal cells)</td>
<td>Cytoplasmic and perinuclear</td>
</tr>
<tr>
<td>1</td>
<td>&lt;5% stained cells (weak labelling)</td>
<td>&lt;5% stained parabasal or intermediary cells</td>
<td>&lt;5% intranuclear staining (fibroblasts and epidermis)</td>
</tr>
<tr>
<td>2</td>
<td>5–10% stained cells (variable, moderate labelling)</td>
<td>&gt;10% stained parabasal cells (moderate labelling)</td>
<td>5–10% intranuclear staining (moderate labelling)</td>
</tr>
<tr>
<td>3</td>
<td>10–20% stained cells (strong labelling)</td>
<td>10–20% stained parabasal or intermediary cells (strong labelling)</td>
<td>5–10% intranuclear staining (strong labelling)</td>
</tr>
<tr>
<td>4</td>
<td>&gt;20% stained cells (strong labelling)</td>
<td>&gt;20% stained cells in all the epidermal layers (strong labelling)</td>
<td>&gt;10% intranuclear staining (strong labelling)</td>
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RESULTS

Fibrosis

Picrosirius Red stained cryosections were graded histologically for fibrosis, as indicated above (Fig. 1A–E).

Before SOD treatment (MO), 12 of the 42 (29%) irradiated skin samples exhibited grade 4 fibrosis, 22 (52%) grade 3, 7 (17%) grade 2, wound healing-type, fibrosis; only one patient irradiated 19 years previously, a patient aged 50 years at time of irradiation, had a subnormal dermis (grade I), with only slight thickening of the connective tissue. No sample was classified as grade 0 (normal dermis).

The OD/cm² of the corresponding dye eluates were correlated with histological grading (Fig. 2). Histological grades 1, 2 and 3 correlated well with 2, 3 and 5 OD values. For histological grade 4, the OD values were more dispersed, possible as the result of a morphological under-evaluation of high-grade fibrosis.

Six months after the start of SOD treatment, an objective but variable response was observed in 31 of the 42 (74%) patients (Fig. 3). Eleven out of 12 (92%) patients with grade 4 fibrosis before SOD showed a mean decrease in the histological grade of about 50%. No significant correlation was found between response intensity and time elapsed between radiotherapy and SOD treatment. In one case of initial grade 4 fibrosis, the dermis had a normal histological appearance, a normal cellularity and collagen distribution at 6 months; the patient, 60 years old, had been irradiated 33 years previously and this long interval did not reduce her capacity to respond to SOD. Patients with an initial grade 3 fibrosis showed a decrease in 18 out of 22 (82%) patients after SOD treatment. In one case of initial grade 4 fibrosis, the dermis had a normal histological appearance, a normal cellularity and collagen distribution at 6 months; the patient, 60 years old, had been irradiated 33 years previously and this long interval did not reduce her capacity to respond to SOD. Patients with an initial grade 3 fibrosis showed a decrease in 18 out of 22 (82%) patients after SOD treatment.

In this group, the mean decrease in fibrosis grade was about 40%. One patient, 47 years old at the time of radiotherapy (¹⁰⁰Co, 45 Gy), treated topically by SOD 19 years after irradiation, showed a total disappearance of dermal fibrosis. Seven cases had grade 2 fibrosis before treatment. Only 2 out of 7 (16.6%) patients displayed an objective response. In one case of initial grade 1, no change was observed after SOD treatment. The overall decrease of fibrosis grading after SOD treatment was on average 37%.

No change was observed in 11 out of 42 (26%) patients: 1 grade 4, 4 grade 3, 5 grade 2 and 1 patient with a dermal fibrosis grade 1 before treatment. There was no significant correlation between responses to treatment and age at the time of radiotherapy or of SOD treatment, nor with the time elapsed between irradiation and the start of SOD treatment (data not shown).

Modifications of dermal fibroblast phenotype by SOD

Vimentin and 5β-hydroxilase labelling allowed specific identification of dermal fibroblasts. SMC-α actin microfilaments, observed in irradiated dermal fibroblasts, defined a myofibroblastic, 'activated' phenotype. Figure 4 summarizes the immunohistochemical data. SMC-α actin cytofilaments were visualized in fibroblasts, at times varying from 3 months to 40 years after irradiation. Forty out of 42 biopsies (95%) were graded 1 (27) or 2 (13) for SMC-α actin positivity. In two patients, irradiated 12 and 3 years before SOD treatment, with a skin dose of about 78 Gy and 55 Gy, respectively, numerous myofibroblasts were observed (grade 3).

Unexpectedly, the number of activated fibroblasts and the staining intensity were increased in 33 cases (79%), 3 months after completion of SOD treatment. No change was noted in 8 cases and 1 case showed a weaker staining, without correlation with age or other radiotherapy parameters. Before treatment the immunohistochemical staining was relatively weak (mean grade ± SD: 1.5 ± 0.6). After 6 months 79% of patients showed an increase in the number of labelled dermal fibroblasts and the mean grade for SMC-α actin was 2 ± 0.7 (mean ± SD). The mean score increased from 1.452 to 2.024 and the mean variation after 6 months follow-up was + 0.561 (P < 0.001).
Fig. 1—Histochemical grading of skin radiofibrosis. Five grades (A: grade 0; B: grade 1; C: grade 2; D: grade 3; E: grade 4) were defined on the basis of accumulation and thickening of collagen fibres in the dermal connective tissue and around the vessels (Sirius Red, Harris haematoxylin counterstain, × 120).
EGFR and irradiated epidermal cells

An overexpression of EGFR along the epidermal cell layers was observed on the initial biopsies. However, the immunolabelling was weak and limited to the intermediary layer when the time elapsed between irradiation and initial punch biopsy was over 5 years. In 40 out of 42 patients (95%) the labelling was graded 1 (27) or 2 (13). After treatment, we observed an increase in the immunolabelling in 28 out of 42 patients (67%): graded 1 in only 9 cases (21%), 2 in 21 cases (50%), 3 in 10 cases (24%) and 4 in the last 2 cases (5%). No change was observed in 14 samples after treatment (Fig. 5).

The mean histochemical grade of EGFR was $1.5 \pm 0.5$ before SOD. At 6 months 67% of cutaneous biopsies...
The documentation of the histological effects of SOD treatment in radiation-induced skin fibrosis required special care. Skin biopsies were obtained before and after treatment from cutaneous areas exposed to similar radiation doses. To avoid technical variations from one assay to the other, skin cryosections of samples taken before and after treatment were deposited on the same slide and stained at the same time. Quantitative spectrophotometric measurement of collagen accumulation, using Sirius Red, permitted a more precise classification of morphological features. This study concerned 42 patients, who were also evaluated clinically (results to be reported elsewhere). In 11 patients, no histological change was observed 3 months after the end of SOD treatment. Seven out of 11 (64%) patients reported a decrease of pain. In 36% of them the thermographic planimetry showed a decrease in the area of fibrosis. The present data confirm that SOD decreases collagen deposition in the extracellular matrix of irradiated dermis in 74% of treated patients. These results were observed 3 months after the completion of a 3-month treatment. Patients will be followed for several years in order to determine whether the improvement is stable or not. No correlation was found between the level of improvement and the delay between irradiation and SOD treatment. This conflicts with previous clinical studies. In the present series, a significant SOD-induced reduction in fibrosis grade was observed in patients irradiated as long ago as 40 years previously. On the other hand, some patients, irradiated less than 3 years before treatment, did not show any change after SOD treatment.

The immunohistochemical study concerned 3 parameters. TGFβ1, which is known to play an essential role in the turnover of extracellular matrix, and which expression is altered in irradiated skin (increased intensity and nuclear localization), compared to normal skin, was not affected by SOD treatment. Myofibroblasts are defined as reactive...
cells which appear in injured connective tissue (wound or burn healing, irradiation, auto-immune pathology, etc.). They express SMC-α actin microfilaments, which are observed early (few weeks) in dermal irradiated fibroblasts and persist for long periods (40 years in our series). SOD treatment of skin radiofibrosis frequently resulted in an increase of SMC-α actin expressing fibroblasts. An experimental in vitro model would be useful to study the changes of the myofibroblastic phenotype with time, γ-ray doses and SOD treatment. EGFR has been reported to be overexpressed in the epidermis, as early as 1 month after γ-irradiation.21 The immunohistochemical expression decreased in the present study after 5 years, but increased following SOD treatment.

The therapeutic effects of SOD on radiofibrosis are histologically objective and significant, but the molecular mechanisms involved after irradiation remain unknown. The immunohistochemical data reported in this study imply that some fibroblastic and epidermal cellular functions are ‘reactivated’ by the enzyme and that skin radiation-induced fibrosis can be reverted.

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References


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