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Amifostine Does Not Prevent Activation of TGFβ1 but Induces smad 7 Activation in Megakaryocytes Irradiated in Vivo

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Experiments were undertaken to assess the role of amifostine in the activation of latent TGFβ1 and in the smad proteins cascade (smad 2/3, smad4, smad7), focusing on megakaryocytes, in the bone marrow irradiated in vivo. Non-irradiated megakaryocytes were negative for active TGFβ1. Immunopositivity to active TGFβ1 was detected in megakaryocytes 10 days after irradiation in amifostine- treated and untreated marrows. Smad 2/3 and smad 4 were strongly positive in the nucleus of megakaryocytes 10 days after irradiation. At the same time, a predominant hypocellular bone marrow with foci of hematopoiesis was observed with few megakaryocytes. An increase in the number of reticulin fibers was also seen. In amifostine-treated marrows, smad 2/3 and smad4 were not detected in the nucleus but were positive in the cytoplasm of megakaryocytes 10 days after irradiation. Coincidentally, bone marrows were cellular with megakaryocytes. Smad7 immunoexpression was detected in the cytoplasm of megakaryocytes in the non-irradiated, amifostine-treated and in the irradiated, amifostine-treated marrows. Data indicate that amifostine does not prevent latent TGFβ1 activation in irradiated megakaryocytes. While TGFβ1 signal transduction occurs in megakaryocytes in untreated bone marrows, it is inhibited in megakaryocytes in amifostine-treated marrows due to the induction of smad 7 activation. This is the first report showing smad 7 activation by amifostine. Our results also suggest a role for TGFβ1 as an inhibitor of megakaryocytes in vivo. Am. J. Hematol. 71:143–151, 2002. © 2002 Wiley-Liss, Inc.

Key words: bone marrow; amifostine; γ rays; TGFβ; smad cascade

INTRODUCTION

The bone marrow, following irradiation, is an acute responding tissue. It can be severely injured after accidental exposures to radiation and also constitutes a critical organ in the interaction between drugs and radiation during the treatment of malignant neoplasias, sometimes limiting such treatment [1,2]. Depending on the volume of irradiated bone marrow and the radiation dose, a pancytopenia, primarily leucopenia and thrombocytopenia, can occur in the peripheral blood due to the injury to stem cells [1,2], resulting in infection, bleeding, and anemia caused by hemorrhage [3]. Myelofibrosis can also develop as a late effect of radiation [4]. Therefore, protection of the bone marrow cells is highly desired in clinical situations.

Amifostine, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid, is an aminothiol phosphorothioate which contains phosphate moieties covering the sulfhydryl

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group in order to prevent the oxidation seen with free thiol groups. It cannot be transported across the cell membrane until the phosphate group is cleaved. This is accomplished enzymatically by alkaline phosphatase, which is membrane bound in many cells, particularly in normal tissues. Amifostine has been found to protect preferentially normal tissues, as opposed to tumors against radiation injuries [6], and bone marrow is one of the most protected organs [7–9]. In the gastrointestinal tract, amifostine has been found to protect from radiation-induced fibrosis [10].

There are several hypotheses for the mechanisms by which amifostine protects against radiation and chemotherapy toxicities, these include oxygen depletion [6,11], hydrogen atom donation [11], enhancement of cell cycle arrest [12], and scavenging of free radicals [13]. However, these mechanisms have not yet been clearly established.

In physiological conditions, hematopoietic stem cells undergo self-replication, proliferation, and differentiation into mature blood cells. All these processes are regulated by stimulatory and inhibitory hematopoietic growth factors [14].

Transforming growth factor β (TGFβ) is a multifunctional regulator of cell proliferation and differentiation, and it has been found to be one of the cytokines involved in the regulation of hematopoiesis [15,16]. Present in mammals as three distinct isoforms, TGFβ1, TGFβ2, and TGFβ3 [17], the TGFβ proteins are synthesized as inactive protein precursors with the latency associated peptide (LAP) [18]. The activation occurs after heat or acid treatment [19], plasmin proteolysis [20], deglycosylation [21], binding to thrombospondin [22], or oxidative stress [23], when TGFβ is released from LAP. In general, mammals possess receptors to TGFβs and the peptide regulates cell function via paracrine and autocrine systems [17].

A group of structurally related proteins, referred to as smads, has been shown to transduce signals downstream from TGFβ family receptors. The first member of the smad family was identified in a genetic screen for modifiers of decapentaplegic (dpp), the ortholog fly of bone morphogenic protein 2/4 (a member of TGFβ family), in *Drosophila.* This smad was originally named Mothers against dpp (Mad) and encodes a protein that transduces signals downstream of dpp. The vertebrate ortholog of Mad, smad 1, as well as a number of other smads that function downstream from distinct TGFβ family members, have since been identified [24,25]. When TGFβ is released from LAP, it binds to type II serine/threonine kinase receptor (TβRII) which phosphorylates TβRI. After activation by TβRI, smad 2 and smad3 transiently interact with and become phosphorylated [26]. They then oligomerize with the common mediator, smad 4 [27]. The hetero-oligomeric complex is then translocated to the nucleus and regulates the transcription of target genes [28]. The inhibitory smads, smad6 and smad7, form a stable interaction with the activated receptor type I (TβRI) and prevent binding to and activation of smad2 and smad3 [29]. Smad 7, in particular, was found to be predominantly localized in the nucleus and accumulates in the cytoplasm following TGFβ receptor activation [30].

In the bone marrow, TGFβ was found to inhibit the proliferation and differentiation of murine and human early hematopoietic progenitors, in vitro, and appears to stimulate the growth of less primitive progenitors [7,31]. It regulates the growth of human marrow fibroblasts as well as collagen and fibronectin synthesis [32].

TGFβ was detected immunohistochemically in megakaryocytes and some mononuclear cells [33]. Peripheral blood monocytes and neutrophils have been found to produce TGFβ1 [34]; however, megakaryocytes are a major site of synthesis and storage of TGFβ [35]. It has been shown that mature megakaryocytes synthesize TGFβ1 and package it intracellularly into α-granules [35]. In platelets, TGFβ was found stored in the latent form [35,36].

TGFβ has been shown to be a potent inhibitor of megakaryocytes in vitro [37,38], but its role in megakaryocytic homeostasis has not yet been established in vivo [35]. TGFβ was also found increased in some hematological diseases, such as myelofibrosis [39], and megakaryocytes are suggested to be one of the possible sources of the proteins [40,41]. For these reasons we focused on megakaryocytes in the present study.

It has been shown that radiation activates latent TGFβ and radiation-induced fibrosis [42,43]. Induction of TGFβ was detected in 1 hr and also 3 and 7 days after irradiation in mammary gland cells. This increase was related to collagen III remodeling [42,44]. Induction of TGFβ protein and mRNA were detected in skin 6 hr after irradiation [43]. In the bone marrow, in vivo, no significant increase in TGFβ mRNA was detected after irradiation [45]. However, regulation at the TGFβ protein activation level could occur without regulation at the level of transcription or translation [43]. It is suggested that the rapid latent TGFβ activation after irradiation is due to radiation generated reactive oxygen [23,44]. The continued elevation is suggested to be cell-mediated, possibly via plasmin [23,44].

Experiments have shown that latent TGFβ proteins are redox sensitive [23,44]. TGFβ1 induces the release of hydrogen peroxide (H_2O_2) from human lung fibroblasts [46]. The expression of antioxidative enzymes such as manganese-superoxide dismutase, copper, zinc-superoxide dismutase, and catalase were suppressed by TGFβ1 in hepatocytes of rats [47]. The overall intracellular oxidized state of mouse osteoblastic cell line was increased after addition of TGFβ, and this increase was

abolished by the addition of radical scavengers such as catalase and *n*-acetylcysteine [48]. Thiol modulation of TGFβ1 antiproliferative effects in endothelial cells has also been reported [49]. When cysteine, cysteamine, and *n*-acetylcysteine were added to endothelial cell cultures, TGFβ1 inhibition of radioactive thymidine (TdR) uptake and also the inhibition of cell proliferation were attenuated [49].

In the present study, we used immunohistochemistry to assess the role of amifostine in the activation of latent TGFβ1 and in the smad proteins cascade (smad 2/3, smad 4, and smad 7), focusing on megakaryocytes, in the bone marrow irradiated in vivo.

METHODS AND MATERIALS

Animals

Eighty-four (84) adult male C57Bl mice (20–24 g) were used. The study was approved by the Federal University of São Paulo Board for Laboratory Animals.

During experiments, the mice were kept in plastic cages on sawdust, with chow and tap water available ad libitum. Animals were randomly assigned to the following groups.

Ami$^-$/rad$^-$. Eighteen non-irradiated mice received 0.5 mL of a 0.9% physiological saline solution (PSS) intraperitoneally (i.p.). They were assigned into six subgroups with three animals each and sacrificed by cervical dislocation at 4, 12, and 24 hr and 10, 30, and 90 days after injection.

Ami$^+$/rad$^-$. Eighteen non-irradiated mice received amifostine (400 mg/kg i.p. supplied by US Bioscience), freshly dissolved in double-distilled water. Mice were assigned into subgroups and sacrificed at the same intervals as in group 1.

Ami$^-$/rad$^+$. Twenty-four mice received 0.5 mL of PSS i.p. 30 min before a single whole-body radiation dose of 7 Gy. They were assigned in six subgroups and sacrificed 4 hr ($n = 3$), 12 hr ($n = 3$), 24 hr ($n = 3$), 10 days ($n = 5$), 30 days ($n = 5$), and 90 days ($n = 5$) after irradiation.

Ami$^+$/rad$^+$. Twenty-four mice received 0.5 mL of an aqueous solution of 400 mg/kg amifostine i.p., 30 min prior to irradiation and were assigned into subgroups and sacrificed at the same intervals as in group 3.

Radiation Factors

A ^{60}Co irradiation source (Teletherapy machine-Alcyon II, CGR) with a dose rate of 1.35 Gy min^{-1} was used. The mice received a single whole-body radiation dose of 7 Gy. During irradiation, the animals were placed in a special 20 × 20 wooden box with a plastic cover and internal divisions for immobilization, without anesthesia.

Bone Marrow

The left femora were removed, fixed in formol + base 5, decalcified, and embedded in Historesin, for morphology and histometry. The right femora were fixed in phosphate-buffered formalin and after decalcification were embedded in synthetic paraffin for immunohistochemistry. Sections 3 μm thick were cut, and slides were prepared. All slides were stained with Giemsa for morphological analysis. The slides from the periods of 10, 30, and 90 days after injection and/or radiation were also stained for reticulin fibers using the Gomori silver method.

Histometry

Histometry was performed in all subgroups at 10, 30, and 90 days (recovery phase) after treatment, in sections stained with the Gomori silver method to assess the number of reticulin fibers. Coded slides were scored blindly. The number of reticulin fibers were counted using an 100-hit integrated lens (Carl Zeiss KF 10×/18) in the light microscope (Olympus BX40F-3). Ten (10) high-powered (×100) fields were randomly selected. One-hundred hits were counted in each field (total of 1,000 hits per slide). Each hit was scored for the presence of cell and reticulin fiber. The number of reticulin fibers (relative to the 1,000 hits) examined was calculated for each animal. The results were submitted to statistical analysis [50,51].

Statistical Analysis

The Kruskall–Wallis test [52] was used to compare the results of reticulin fiber counting 10, 30, and 90 days after injection and/or radiation. This test was set separately for each group (Ami$^-$/rad$^-$, Ami$^+$/rad$^-$, Ami$^-$/rad$^+$, and Ami$^+$/rad$^+$). To compare the number of reticulin fibers between irradiated amifostine-treated (Ami$^+$/rad$^+$) and untreated (Ami$^-$/rad$^+$), groups the Mann–Whitney test was used. Standard error (SE) was also calculated [52], and P values ≤ 0.05 were considered statistically significant.

Antibodies

The following primary antibodies were used: goat polyclonal immunoglobulin IgG antihuman LAP (TGFβ1) (R&D Systems Inc., Minneapolis, MN), rabbit polyclonal IgG anti mouse, rat, and human TGFβ1 (Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal IgG anti mouse, rat, and human smad 2/3 (Santa Cruz Biotechnology), rabbit polyclonal IgG anti mouse, rat, and human smad 4 (Santa Cruz Biotechnology), and a goat polyclonal IgG anti mouse, rat, and human smad 7 (Santa Cruz Biotechnology).

Immunohistochemistry

Immunohistochemistry was performed by an indirect three-stage immunoenzymatic procedure. Serial slides of 3 μm were mounted on 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO) precoated slides. After deparaffinization, the sections were incubated overnight with the primary antibodies (1:100) at room temperature.

After a wash step with phosphate-buffered saline (PBS), the sections were incubated with the biotinylated secondary antibodies (1:250) for 3 hr. Subsequently, an Extra-avidin® conjugated with alkaline phosphatase (Sigma) to detect latent TGFβ1, active TGFβ1, smad 2/3, smad 4, and smad 7 was added for 2 hr. To display alkaline phosphatase activity the slides were incubated with substrate using a mixture of naphthol AS-MX phosphate, levamizole, and a chromogenic salt diluted in veronal acetate buffer (pH 8.3). The immunoglobulin complexes were demonstrated by fast red TR (Sigma), resulting in a red positivity for latent TGFβ, active TGFβ1, smad 2/3, smad 4, and smad 7. The sections were then washed in tap water overnight, counterstained with Gill hematoxylin, and mounted with Immu-mount (Shandon, Pittsburgh, PA). Controls with omission of the primary antibody were included in all experiments.

The immunohistochemistry procedure to detect latent and active TGFβ1 was performed in 3 animals from each subgroup sacrificed 4, 12, and 24 hr and 10, 30, and 90 days after treatment. The detection of smad 2/3, smad 4, and smad 7 was performed in 3 animals from Ami$^-$/rad$^-$, Ami$^+$/rad$^-$, Ami$^-$/rad$^+$, and Ami$^+$/rad$^+$, sacrificed 10 days after treatment.

RESULTS

Morphological Changes: Comparison Between Irradiated Amifostine-Treated (Ami$^+$/rad$^-$) and Untreated (Ami$^-$/rad$^+$) Marrows

The bone marrow sections of the irradiated amifostine-treated animals (Ami$^+$/rad$^+$) demonstrated less injured cells 4,12, and 24 hr after irradiation compared to the marrows of irradiated and untreated animals (Ami$^-$/rad$^+$). Ten days after irradiation, amifostine-treated marrows (Ami$^+$/rad$^+$) were cellular with megakaryocytes, while the untreated ones (Ami$^-$/rad$^+$) had foci of cellularity with megakaryocytes and a predominant hypocellularity. In this time period (10 days) the number of reticulin fibers was significantly increased in Ami$^-$/rad$^+$ compared to the Ami$^+$/rad$^+$ group (59/16.4 = 3.5-fold) (Fig. 1). Thirty and 90 days after irradiation, recovery back to normal levels occurred in both Ami$^+$/rad$^+$ and Ami$^-$/rad$^+$ groups.

Immunohistochemical Analysis

TGFβ1. In all non-irradiated bone marrows, at all time periods studied, amifostine-treated (Ami$^+$/rad$^-$) or not (Ami$^-$/rad$^-$), megakaryocytes demonstrated a particularly strong immunopositivity to *latent TGFβ1* (Table I). However, in both groups (Ami$^+$/rad$^-$ and Ami$^-$/rad$^-$), megakaryocytes did not show any positivity to *active TGFβ1* (Table I, Fig. 2A). After irradiation, 4-, 12-, and 24-hr megakaryocytes showed the same pattern of staining as in the controls for *latent and active TGFβ1,* in both amifostine-treated (Ami$^+$/rad$^+$) and untreated (Ami$^-$/rad$^+$) groups. Ten days after irradiation, megakaryocytes, in amifostine-treated (Ami$^+$/rad$^+$) and those present in the foci of hematopoiesis in untreated marrows (Ami$^-$/rad$^+$), were positive, faintly positive, and negative to *latent TGFβ1,* respectively. At the same time, megakaryocytes displayed *active TGFβ1* immunopositivity in both (Ami$^+$/rad$^+$) and (Ami$^-$/rad$^+$) groups (Table I, Fig. 2B). Thirty days after irradiation, in amifostine-treated (Ami$^+$/rad$^+$) or not (Ami$^-$/rad$^+$) groups, megakaryocytes showed the same pattern of staining as seen at 10 days for *latent and active TGFβ1.* Ninety days after irradiation, in both

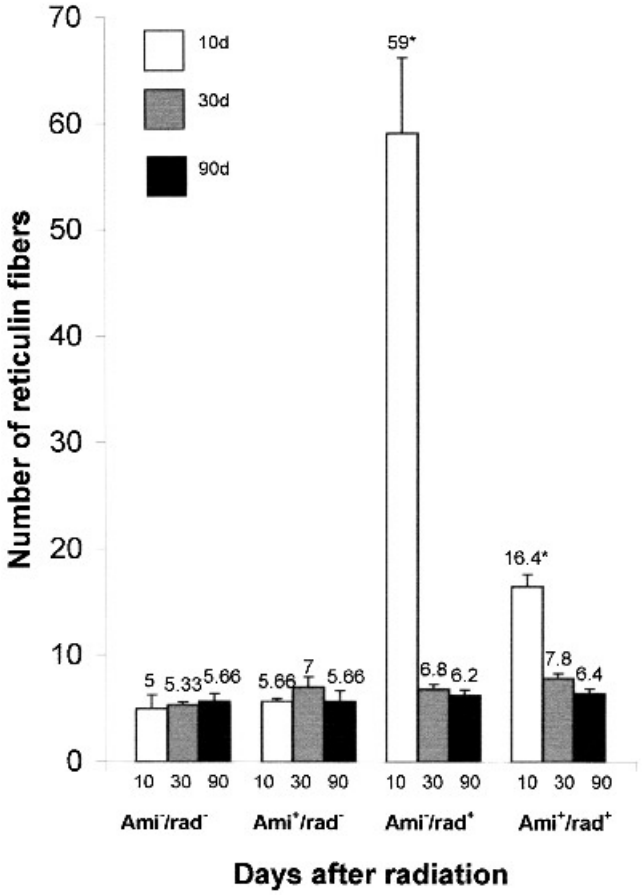

Fig. 1. Number of reticulin fibers in (Ami$^-$/rad$^-$), (Ami$^+$/rad$^-$), (Ami$^-$/rad$^+$), and (Ami$^+$/rad$^+$) groups 10, 30, and 90 days after treatment. Data represent the mean ± SE of values obtained from three (*n* = 3) (Ami$^-$/rad$^-$ and Ami$^+$/rad$^-$) and five (*n* = 5) (Ami$^-$/rad$^+$ and Ami$^+$/rad$^+$) animals per experimental group. *$P \leq 0.05$.

TABLE I.[1]

Proteins	Ami⁻/rad⁻	Ami⁺/rad⁻	Ami⁻/rad⁺	Ami⁺/rad⁺
Latent TGFβ1	+++	+++	++, +, –	++, +, –
Active TGFβ1	–	–	++	++
Smad 2/3	cyto: ++	cyto: ++	nu: +++	cyto: ++
Smad 4	cyto: +	cyto: +	nu: +++	cyto: +
Smad 7	nu cyto: –	cyto: ++	nu: +++	cyto: +++

[1]Key: +++, strong positivity; ++, positive; +, faint positivity; –, negative; nu, nucleus; cyto, cytoplasm.

Ami⁺/rad⁺ and Ami⁻/rad⁺ groups, megakaryocytes were predominantly positive to *latent TGFβ1* and faintly positive and negative for *active TGFβ1*.

Smad 2/3, smad 4, and smad 7 (10 days). After observing TGFβ1 activation in megakaryocytes 10 days after irradiation both in amifostine-treated (Ami⁺/rad⁺) and untreated bone marrows (Ami⁻/rad⁺), we assessed the transduction of the TGFβ signal, particularly in megakaryocytes, during this time period.

In the non-irradiated marrows, whether they were amifostine-treated (Ami⁺/rad⁻) or not (Ami⁻/rad⁻), megakaryocytes showed positivity to smad 2/3 and a weak positivity to smad 4 in the cytoplasm (Table I). Immunopositivity to smad 7 was not detected in the cytoplasm or in the nucleus of the non-irradiated and untreated megakaryocytes (Ami⁻/rad⁻) (Table I, Fig. 2E). Amifostine-treated non-irradiated megakaryocytes (Ami⁺/rad⁻) showed immunopositivity to smad 7 in the cytoplasm (Table I, Fig. 2F).

Ten days after irradiation, in untreated marrows (Ami⁻/rad⁺), smad 2/3, smad 4 (Table I, Fig. 2C,D), and smad 7 (Table I, Fig. 2H) were strongly expressed in the nucleus of megakaryocytes. At the same time, a predominant hypocellular bone marrow with foci of hematopoiesis and an increase in the number of reticulin fibers was observed. In amifostine-treated marrows (Ami⁺/rad⁺), smad 2/3 and smad4 were not detected in the nucleus but were positive in the cytoplasm of megakaryocytes 10 days after irradiation (Table I). In addition, a strong immunopositivity to smad 7 was detected in the cytoplasm of megakaryocytes in this group (Table I, Fig. 2G). At the same time, bone marrows showed a better cellularity compared to the untreated ones. These results suggest that TGFβ1 signal transduction is occurring in megakaryocytes in untreated marrows (Ami⁻/rad⁺) while in the amifostine-treated (Ami⁺/rad⁺) ones it is inhibited in megakaryocytes due to the activation of smad7. The data also show that amifostine activates smad 7 in megakaryocytes (Ami⁺/rad⁻).

DISCUSSION

The radiation injuries in bone marrow cells were attenuated at all time periods by amifostine, supporting the findings of other studies [7–9]. It was also shown that the number of reticulin fibers was lower 10 days after irradiation in Ami⁺/rad⁺ compared to the (Ami⁻/rad⁺) group. Amifostine has been found to protect the gastrointestinal tract of mice from late effects of radiation such as ulceration, fibrosis, and vascular changes by a protection factor of 1.3 [10]. In the present study, the significantly lower amount of reticulin fibers seen 10 days after irradiation in Ami⁺/rad⁺ compared to the Ami⁻/rad⁺ group is also consistent with amifostine protection against reticulin fibrosis in the bone marrow. Several hypotheses have been suggested to explain amifostine radioprotection [6,11–13], but its mechanisms are not completely understood.

The expression of latent TGFβ1 was detected immunohistochemically in a representative amount of megakaryocytes in all non-irradiated, amifostine-treated (Ami⁺/rad⁻) or non-treated (Ami⁻/rad⁻) bone marrows. These findings are consistent with other reports in the literature, which show the presence of TGFβ1 in megakaryocytes and platelets [35,36]. However, the expression of active TGFβ1 was not detected in megakaryocytes, in the non-irradiated bone marrows (Ami⁻/rad⁻ and Ami⁺/rad⁻).

TGFβ is a known modulator of hematopoietic cells [53] and megakaryocytes are the major site of synthesis and storage of TGFβ1 [35]. In platelets, TGFβ was found stored in the latent form [35,36]. It has been suggested that the fate of TGFβ1 produced by megakaryocytes is packaging into platelet α-granules for use primarily in wound healing [35]. The platelet latent complex has also been postulated to be a "delivery" complex, extending the half life of the protein and insuring that TGFβ1 acts in target cells capable of activating the latent form [36]. Considering these aspects we would expect to find a positivity to latent TGFβ1 in megakaryocytes in physiological conditions.

During the bone marrow injury phase, 4, 12, and 24 hr after irradiation, in (Ami⁺/rad⁺ and Ami⁻/rad⁺) marrows, the pattern of latent and active TGFβ1 expression in megakaryocytes was the same as seen in the non-irradiated controls (Ami⁻/rad⁻ and Ami⁺/rad⁻). Ten days after irradiation, megakaryocytes showed three distinct patterns of latent TGFβ1 expression in (Ami⁺/rad⁺ and Ami⁻/rad⁺) marrows; positive, intermediary positivity and negative cells. In the same period, amifostine-treated (Ami⁺/rad⁺) and untreated (Ami⁻/rad⁺) megakaryocytes clearly displayed active TGFβ1 immunopositivity. These findings indicate activation of latent TGFβ1 in both amifostine-treated and untreated megakaryocytes 10 days after irradiation and that amifostine does not prevent TGFβ1 activation in megakaryocytes irradiated in vivo. Studies with another antioxidant, superoxide dismutase (SOD), have shown that the compound did not modify the immunoexpression of TGFβ1 in epidermal cells [54].

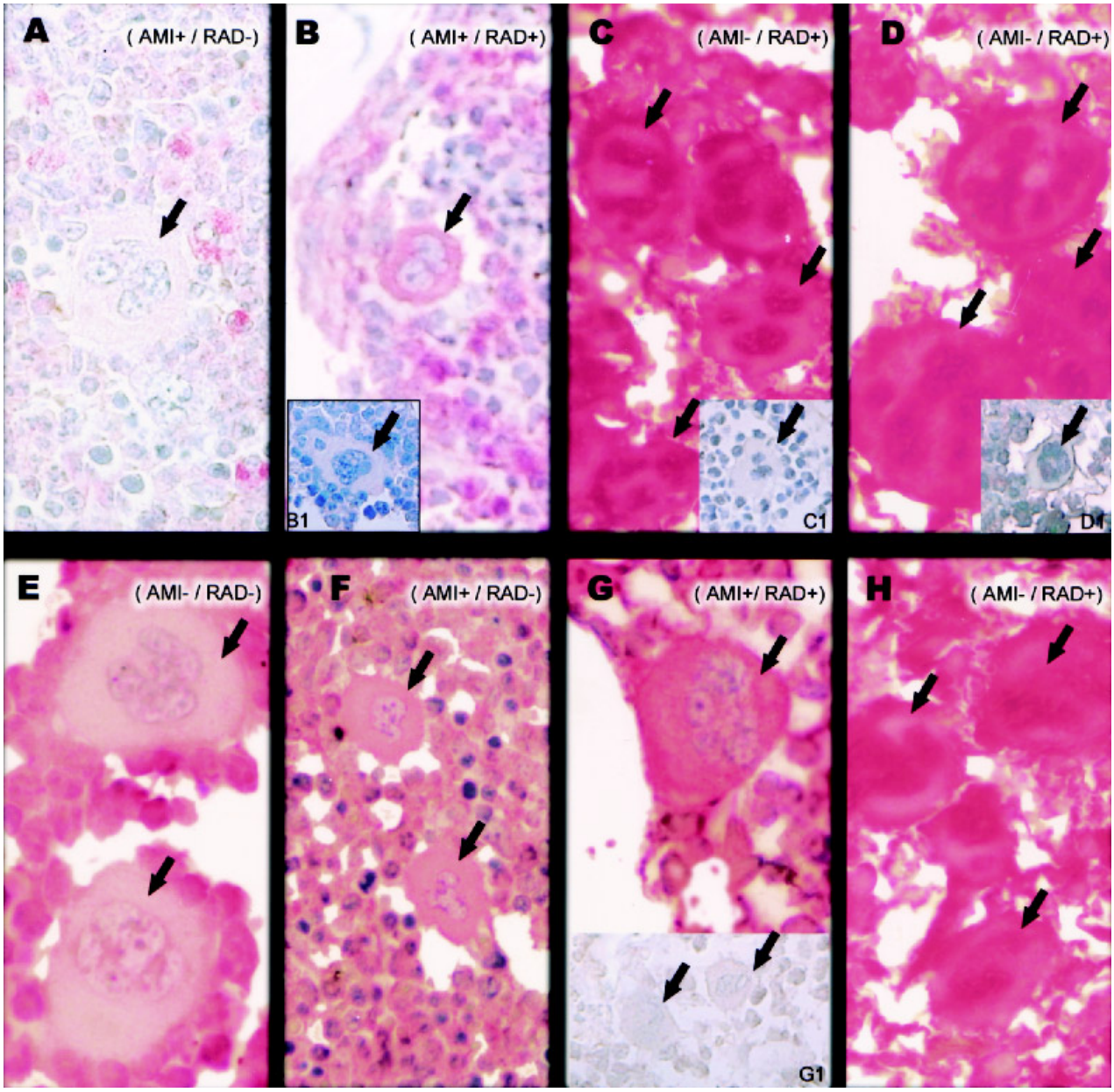

Fig. 2. Non-irradiated and amifostine-treated (Ami^+/rad^-) megakaryocyte (arrow) does not show immunopositivity to active TGFβ1. (A) (magnification ×715) Megakaryocyte immunopositive (arrow) to active TGFβ1 10 days after amifostine + radiation (Ami^+/rad^+). (B) (magnification ×630) Megakaryocytes (arrows) show strong immunopositivity to smad 2/3 (C) (magnification ×670) and to smad 4 (D) (magnification ×680) in the nucleus 10 days after irradiation (Ami^-/rad^+). Non-irradiated and untreated megakaryocytes (arrows) do not show immunopositivity to smad 7 (Ami^-/rad^-). (E) (magnification ×1,050) Megakaryocytes (arrows) showing immunopositivity to smad 7 in the cytoplasm in the non-irradiated and amifostine-treated (Ami^+/rad^-) bone marrow. (F) (magnification ×630) Megakaryocyte (arrow) with strong immunopositivity to smad 7 in the cytoplasm 10 days after amifostine + radiation (Ami^+/rad^+). (G) (magnification ×960) Megakaryocytes (arrows) with strong immunopositivity to smad 7 in the nucleus 10 days after irradiation (Ami^-/rad^+). (H) (magnification ×665) Primary antibodies: polyclonal anti-mouse, rat, and human TGFβ1, smad 2/3, smad 4, and smad 7 immunoglobulin G (IgG, Santa Cruz Biotechnology). Negative controls to active TGFβ1 (B1) (magnification ×550); smad 2/3 (C1) (magnification ×530); smad 4 (D1) (magnification ×530); and smad 7 (G1) (magnification ×540). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

With regard to the Ami^+/rad^+ group, our results support other investigations that have demonstrated TGFβ in blood islands of developing rat fetuses at a time of proliferative activity, when TGFβ was expected to be negative [53]. Data also showed activation at the TGFβ protein level in vivo, although, no significant increase in the levels of TGFβ mRNA after 7.75 Gy has been demonstrated [45].

The detection of latent TGFβ1 activation in megakaryocytes at 10 days, and persisting until 30 days after irradiation, indicates a delayed activation of TGFβ1 in this cell type. In tissues, such as mammary gland cells, TGFβ was found activated within hours and also days after irradiation [42,44]. Early activation was suggested to be the consequence of oxidative stress from the free radicals induced by radiation and the continued elevation, probably due to a cell-mediated mechanism, via plasmin. With reference to our results, we suggest that the delayed radiation-induced activation of latent TGFβ1 detected in megakaryocytes in Ami^+/rad^+ and Ami^-/rad^+ may also be due to a cell-mediated mechanism.

It is also important to mention that 10 days after irradiation, the initial fibrosis was increased in (Ami^-/rad^+) group concomitant with active TGFβ1 positive megakaryocytes. With regard to the cellular sources of active TGFβ1 in the bone marrow, which could stimulate fibrosis, studies of some hematological diseases, such as myelofibrosis, suggest that megakaryocytes are the source [40,41]. However, others have reported that monocytes may be the source of TGFβ1 [39]. In physiological conditions monocytes, neutrophils, and megakaryocytes were found to produce TGFβ1 [34,35]. Therefore, the cellular sources possibly involved in the bone marrow fibrosis are not clearly defined and could be multiple. We suggest that the active TGFβ1 positive megakaryocytes present in the foci of hematopoiesis in irradiated bone marrows (Ami^-/rad^+) may contribute to the process of radiation-induced initial reticulin fibrosis.

After the detection of latent TGFβ1 activation in megakaryocytes 10 days after irradiation, in both Ami^+/rad^+ and Ami^-/rad^+ bone marrows we were interested in the assessment of the signal transduction of TGFβ in this cell type.

The non-irradiated and untreated megakaryocytes (Ami^-/rad^-) showed immunopositivity to smad 2/3 and smad 4 in the cytoplasm. After irradiation, a strong immunopositivity was detected in the nucleus (Ami^-/rad^+). Non-irradiated amifostine-treated megakaryocytes (Ami^+/rad^-) also demonstrated smad 2/3 and smad 4 immunopositivity in the cytoplasm, but after irradiation positivity was not detected in the nucleus (Ami^+/rad^+). These results indicate that the signal transduction via activation of smad 2 and smad 3 by TβRI, oligomerization with smad 4 and translocation of the smad complex to the nucleoli [26–30] is functional in megakaryocytes in the Ami^-/rad^+ group but does not occur in megakaryocytes in Ami^+/rad^+ group.

The immunopositivity to smad 7 was not detected neither in the nucleus nor in the cytoplasm of non-irradiated and untreated megakaryocytes (Ami^-/rad^-). It is possible that under physiological conditions the amount of smad 7 in these cells is too small to be detected immunohistochemically. However, after irradiation, a strong immunopositivity to smad 7 was detected in the nucleus in the (Ami^-/rad^+) group. The expression of inhibitory smad 7 protein was found to be localized within the nucleus in the absence of ligand and translocated to the cytoplasm upon TGFβ1 stimulation where it may act in an autoregulatory negative feedback loop [30]. In addition, smad 7 mRNA expression was reported to be induced by TGFβ1 [29]. Our results showed smad 7 immunopositivity in the nucleus of irradiated and untreated Ami^-/rad^+ megakaryocytes concomitant with positive immunoexpression of active TGFβ1. We suggest that activation of latent TGFβ1 in active TGFβ1 by radiation stimulates smad 7 production in megakaryocytes and the protein accumulates within the nucleus. At the time assessed, smad 7 had not been translocated to the cytoplasm and the autoregulatory negative feedback was not functioning in Ami^-/rad^+ megakaryocytes. Therefore, TGFβ1 signal transduction was occurring in these cells. These findings suggest a role for TGFβ1 as an inhibitor of megakaryocytes. Immunopositivity to smad 7 was detected in the cytoplasm of non-irradiated and amifostine-treated megakaryocytes (Ami^+/rad^-). These results suggest that amifostine itself activates smad 7, which translocates to the cytoplasm since active TGFβ1 was negative in these cells. After irradiation, smad 7 immunopositivity increased in the cytoplasm of amifostine-treated megakaryocytes (Ami^+/rad^-). In this case, amifostine and also active TGFβ1 could have contributed to smad 7 activation. The final result is the inhibition of TGFβ1 signal transduction in megakaryocytes, in irradiated amifostine-treated bone marrows (Ami^+/rad^+).

To our knowledge, this is the first report showing smad 7 activation by amifostine. Recently, it has been reported that interferon γ (IFN-γ), which has been used to treat radiation-induced fibrosis [55,56], induces the expression of smad 7 protein in osteosarcoma cells, thus preventing the cellular response to TGFβ1 [57]. Taken together our data suggest that active TGFβ1 has a role in the inhibition of megakaryocytes in vivo. Results also indicate that amifostine does not prevent latent TGFβ1 activation in irradiated megakaryocytes. Whilst TGFβ1 signal transduction occurs in megakaryocytes in untreated bone marrows (Ami^-/rad^+), it is inhibited in megakaryocytes in amifostine-treated marrows (Ami^+/rad^+) due to the induction of smad 7 activation. It is possible that the inhibition of the TGFβ1 signal transduction cascade, and consequently its inhibitory growth signal could be a mechanism of radioprotection for other bone marrow cells, which contributes to the faster recovery of cellularity induced by amifostine. Our results also suggest that amifostine may be an effective agent for preventing radiation-induced fibrosis in the bone marrow and one possible mechanism for its action could be due to the induction of smad 7 activation.

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