Effect of Ionizing Radiation on the Expression of Transforming Growth Factor-β

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Experiments demonstrate the stimulation of transforming growth factor-β expression in porcine skin soon after γ-irradiation in a dose of 64 Gy and in mouse macrophages after whole body irradiation in a dose of 4 Gy, which suggests the involvement of macrophages that produce transforming growth factor-β during the early postirradiation period in the development of postirradiation fibrosis.

Key Words: *macrophage; transforming growth factor-β; fibrosis*

Local high-dose irradiation of the skin causes acute inflammatory reaction (erythema) followed by chronic inflammation with active fibroblast proliferation and accumulation of the extracellular matrix in the damaged zone. An important role in this process is played by neutrophils and macrophages (MPH) migrating to the inflammatory focus from the blood. MPH actively participate in both destruction and final regeneration processes by synthesizing cytotoxic and growth-stimulating compounds, respectively [1,8]. Among other growth-stimulating factors a principal role in wound healing is played by transforming growth factor-β (TGF-β) due to universal distribution of TGF-β receptors on and regulation of cell migration, growth, and differentiation by TGF-β [11]. TGF-β stimulates the synthesis of collagen, fibronectin, and other components of the extracellular matrix, which allows us to assume that this cytokine plays a key role in the development of fibrosis. This assumption is confirmed by long-term (more than 1 month) TGF-β expression in the skin after local irradiation [7].

A cascade of cell events triggered by radiation and culminating in fibrosis can be considered as a continuous process. In the present study we investigate the expression of TGF-β in porcine skin immediately after local irradiation and in mouse MPH irradiated *in vivo* and *in vitro*. Since the intensity of TGF-β production depends on the activation phase [9], we also assessed cytotoxic and growth-stimulating activity of MPH.

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MATERIALS AND METHODS

The skin of 3 laboratory pigs was locally irradiated using an iridium source as described previously [5]. To this end, 2-cm clamps were fixed to the lateral skin of anesthetized pigs. Radiation dose was controlled using two dosimeter-coupled compartments, it was equal to 64 Gy for each animal.

Peritoneal MPH were isolated from 10-12-month-old C57Bl/6 mice weighing 18-20 g (Rappolovo Nursery, Russian Academy of Medical Sciences).

In series I, the mice were exposed for 179 sec to whole body irradiation in an Igur-1 apparatus (total dose 4 Gy). Peritoneal MPH were isolated from the 1st to the 10th day, cultured for 18 h in 96-well plates in 200 ml RPMI-1640 supplemented with 2% embryonic calf serum (37°C, 5% CO_2), and the conditioned medium was than collected to test their effect on cell proliferation.

In series II, MPH isolated from intact mice were cultured under similar conditions for 18 h and then

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TABLE 1. Expression of TGF-β mRNA in Porcine Skin 6 h after Irradiation in a Dose of 64 Gy

No. of animal	Normal skin		Exposed skin		Relative increase of TGF-β mRNA expression
	TGF-β mRNA	18S mRNA	TGF-β mRNA	18S mRNA	
1	10.24	12.14	15.62	15.43	1.5
2	14.32	16.21	31.05	18.21	2.2
3	5.28	14.48	21.83	16.21	4.1

irradiated for 358 sec with an X-ray apparatus (8 Gy). The supernatants were collected on days 0-10, and their growth-stimulating and cytotoxic activities were assessed *in vitro* using KB (human oral carcinoma) and 10T 1/2 (transformed mouse fibroblasts) cells. All experiments were repeated 5-6 times. Experimental methods were described in detail elsewhere [1,8].

Precultured for 18 h peritoneal MPH were irradiated in doses of 12, 8, 4, 1.2, 0.2, and 0.1 Gy for 537, 358, 179, 53.7, 9, and 4.5 sec, respectively,

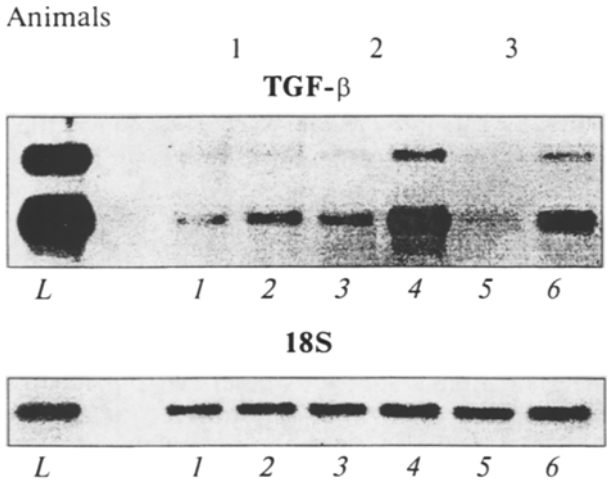

Fig. 1. Northern-blot. Expression of transforming growth factor-β (TGF-β) 6 h after irradiation in a dose of 64 Gy. *2, 4,* and *6*: irradiation; 1, 3, and 5: normal skin. Lymphocyte mRNA (L) was used as positive control of TGF-β expression. Northern blot: 10 μg mRNA; hybridization: ^{32}P-^{33}P labeled TGF-β and 18S DNA probes. 18S mRNA serve as the control of uniform RNA loading.

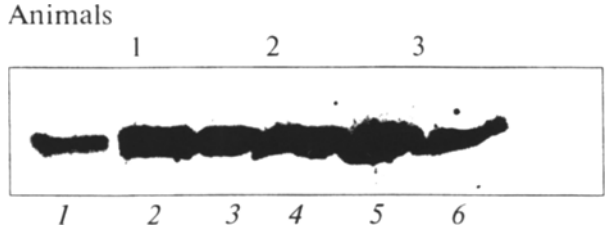

Fig. 2. Western-blot. Synthesis of transforming growth factor-β (TGF-β) 6 h after irradiation in a dose of 64 Gy. *2, 3,* and *5*: irradiation; *1, 4,* and *6*: normal skin (control).

after which 1 μCi ^{3}H-thymidine was added and the samples were processed as described elsewhere.

Surgically dissected irradiated porcine skin was frozen at -80°C, powdered, and homogenized in GIT denaturing solution containing 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, and 0.1 N β-mercaptoethanol (10 ml/mg tissue). The total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method [3]; mRNA was isolated by affinity chromatography of polyadenylated RNA on oligo dT-cellulose (Pharmacia-LKB).

For Northern-blot analysis the isolated mRNA was fractionated in 1% agarose gel containing 2.2 M formaldehyde, transferred onto nitrocellulose filters (Schleicher and Schuell) according to the Southern method, and fixed in Stratalinker-1800. Blot hybridization was performed with Sac I/Pvu II restriction fragments of TGF-β1 labeled by ^{32}P-^{33}P nick-translation (Megaprime RPN 1606 Kit, Amersham). The blots were washed in 1% SSC and 0.1% SDS at 55°C and used for autoradiography. To determine the amount of applied RNA, dehybridization in 0.1% SDS at 100°C followed by rehybridization with 18S ribosomal RNA were performed.

For TGF-β immunoblot assay frozen skin samples were powdered, homogenized in ethanol-acetate (4 ml/g), and processed as described previously [2].

For gel electrophoresis, 10 μg protein extract was mixed with denaturing buffer (1.5 M Tris, pH 8.8; 5% SDS, 0.25% β-mercaptoethanol, and 0.25% glycerol) heated to 100°C, and placed to melting

TABLE 2. Synthesis of TGF-β in Porcine Skin 6 h after Irradiation in a Dose of 64 Gy

No. of animal	Normal skin	Exposed skin	Relative increase in TGF-β synthesis
1	15.51	33.49	2.16
2	31.98	36.01	0.03
3	21.95	47.71	2.17

ice. The proteins were separated by polyacrylamide gel electrophoresis followed by electroelution to nitrocellulose membrane pretreated with phosphate buffered saline (PBS) containing 0.1% Tween and 3% bovine serum albumin for 2 h to prevent non-specific sorption. The membrane was incubated (3 h, room temperature) with anti-TGF-β1 antibodies (R&D Systems) preliminary adjusted to a concentration of 5 ng/ml with 0.1% PBS-Tween containing 3% bovine serum albumin, washed 3 times with PBS containing 0.1% Tween, incubated for 1 h at room temperature with peroxidase-labeled anti-rabbit immunoglobulin adjusted to a concentration of 1 ng/ml with 0.1% PBS-Tween solution containing 3% albumin, washed again, and TGF-βI protein was visualized by the method of catalytic oxidation of luminol (ECL-reaction, Amersham).

RESULTS

The expression of TGF-β gene and production of TGF-β protein in porcine skin irradiated in a dose of 64 Gy increased significantly 6 h after exposure (2.3- and 1.8-fold, respectively, in comparison with the control, Tables 1 and 2, Figs. 1 and 2).

Peritoneal MPH from mice irradiated in a dose of 4 Gy actively produced TGF-β during the first 3 days postirradiation; then TGF-β synthesis sharply

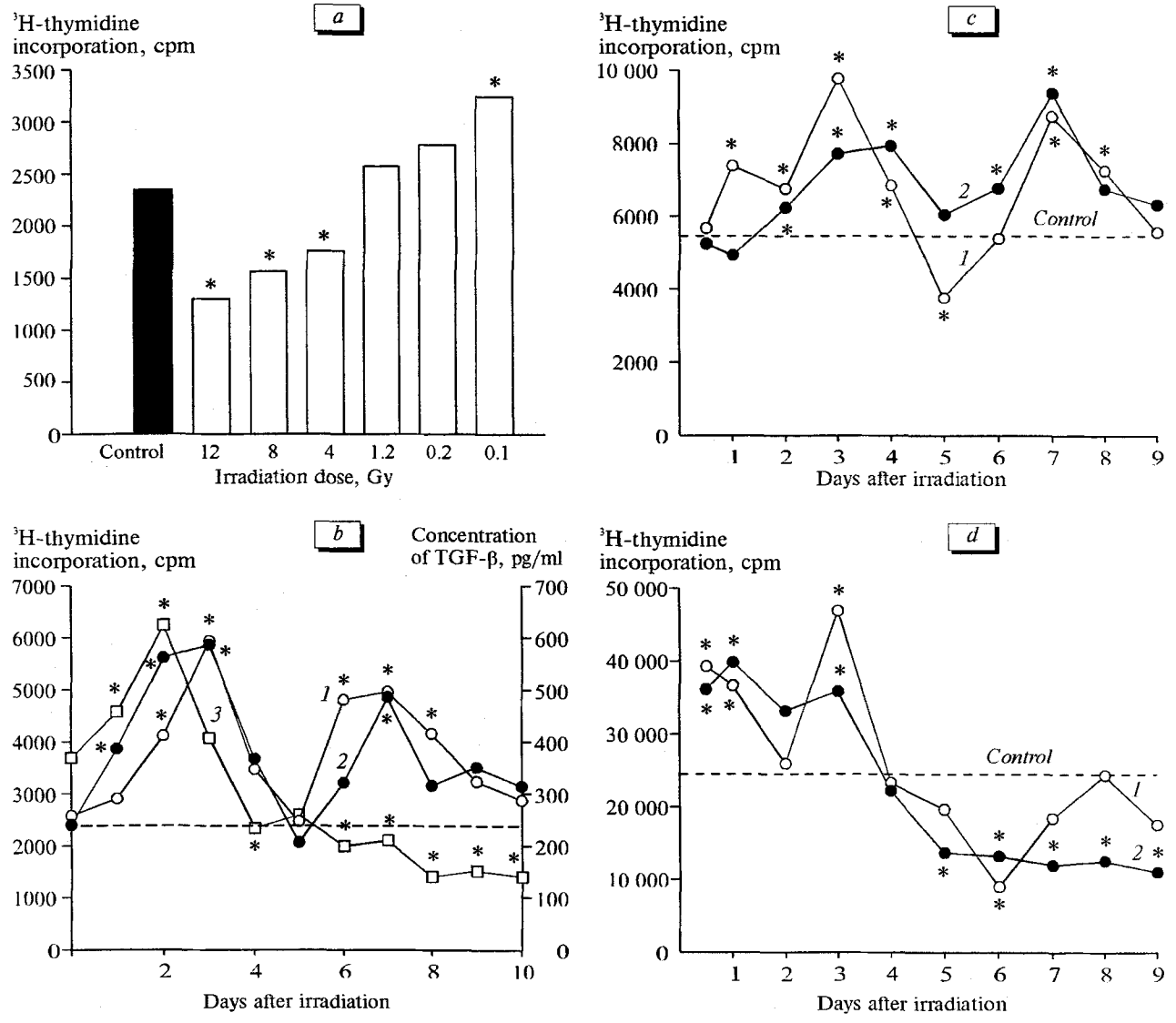

Fig. 3. Proliferative (*a*) and functional activity of mouse peritoneal macrophages irradiated *in vivo* (*b*) and *in vitro* (*c, d*) in doses of 4 and 8 Gy, respectively. Control: intact mouse macrophages (*a*) or complete culture medium containing 2% embryonic calf serum (*b-d*). *b*: KB (*1*) or 10T $^1/_2$ target cells (*2*); TGF-β synthesis by peritoneal macrophages from irradiated mice (*3*) *in vivo*. *c*: 10T 1/2 target cells before (*1*) and after irradiation (*2*); *d*: KB target cells before (*1*) and after irradiation (*2*). *$p<0.05$ compared with the control.

decreased and remained below the control level (Fig. 3, *b*).

We also studied the effect of γ-radiation on MPH proliferation. Irradiation in doses 4 and 8 Gy considerably suppressed MPH proliferation, which probably is an indirect evidence of radiation-induced apoptosis; low doses stimulated cell proliferation. This attests to different mechanisms of cell response to irradiation in a wide dose range (Fig. 3, *a*).

The activity of culture media conditioned by irradiated MPH was studied in two experimental models using human epithelium-derived malignant KB cells and transformed nonmalignant mouse 10T 1/2 fibroblasts. Published data suggest that TGF-β inhibits proliferation of epithelial cells, but this inhibition is reversible and differs from terminal differentiation induced by high calcium doses [4]. We observed no inverse correlation between the concentration of TGF-β in the supernatants and their ability to suppress proliferation of KB cells probably due to the presence of other growth factors in the supernatants or due to low sensitivity of these cells to TGF-β.

It has been shown that TGF-β plays an important role in wound healing. For instance, in human keratinocytes TGF-β stimulates the expression of specific regeneration markers, rather than of the terminal differentiation markers, thus maintaining these cells in an intermediate state allowing other growth factors to abolish growth suppression [6]. In our experiments the media conditioned *in vivo* by irradiated MPH stimulated the growth of KB cells and fibroblasts (2 peaks of stimulation) and exhibited no cytotoxic effect (Fig. 3, *b*).

Our findings agree with published data on the stimulating effect of TGF-β on the proliferation of fibroblasts and other mesenchymal cells [10]. *In vitro* irradiation in a dose of 8 Gy stimulated functional activity of MPH: we observed pronounced alternating peaks of cytotoxic and growth-stimulating activities particularly during the first 5 days postirradiation (Fig. 3, *c*, *d*). We assume that in this experimental model the profile of functional activity of irradiated MPH depends on its initial activity.

Thus, the production of TGF-β by activated MPH *in vivo* and *in vitro* depends on various factors. In our experiments the skin reaction to irradiation greatly varied in different animals.

The synthesis of TGF-β in MPH as soon as during the first few days after irradiation stimulates fibroblast proliferation via the mechanism of feedback autocrine regulation, thus triggering fibrosis in injured skin. TGF-β-induced migration of MPH from the circulation into the focus of inflammation promotes and aggravates these processes.

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