Production of TGF- β1 in Lungs after Low-Dose Whole-Body Radiation Exposure in Fibrosing (C57BL/6) and Non-fibrosing (C3H/J) Mouse Strain

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Abstract. The aim of the presented study was to observe acute and subacute discrete TGF-β1 production after a low-dose whole-body radiation stimulus, known to induce thrombocytopenia. TGF-β1 mRNA production and the number of thrombocytes was followed up in two mouse strains with different tendencies to the origination of fibroses. Mice of the C57BL/6 and C3H/J strains were exposed to a whole-body dose of 7 Gy. Non-irradiated mice of both strains were used as negative controls. The relative number of thrombocytes recorded in lung capillaries was significantly lower in both strains on day 9 after irradiation in comparison with controls. This finding was in accordance with a decrease in the number of thrombocytes in the peripheral blood in irradiated animals of both strains. On day 56 relative platelet counts reached physiological numbers in comparison to controls. On the other hand, TGF-β1 mRNA production was higher in the C57BL/6 strain (on day 9) contrary to minimal production in the C3H/J strain (on day 9) or no production in both groups on day 56 and in controls. Thus, TGF-β1 production without increased thrombocyte trapping in lung vessels in acute stage suggests that an additional mechanism is involved in low-dose radiation-induced cytokine synthesis in lung tissue besides the release of growth factors from thrombocytes.

Several works from the last years demonstrate a considerable contribution of the transforming growth factor β (TGF-β) to initiating, particularly to closing the *circulus vitiosus* in chronic fibrosing processes (Rubin et al., 1995; Grande, 1997). Under physiological conditions, this cytokine participates in the growth of cells as well as tissues and in their differentiation in the course of many regeneration and repair processes. It exerts two-way effects – stimulating and inhibiting. The stimulating effect is particularly aimed at directing the synthesis of fibrillar components of extracellular matrices (collagen I, collagen IV, fibronectin, etc.) (Grande et al., 1993), at increasing the expression of integrins and at stimulation of the "plasminogen activator inhibitor" as well as "tissue inhibitor of matrix metalloproteinases". TGF-β exerts chemotactic effects on monocytes and fibroblasts by stimulating tumour-necrosis factor α (TNF-α) and interleukin 1 (IL-1) production in monocytes. On the other hand, the inhibiting effect of TGF-β is expressed by reduced proliferation of epithelial cells, osteoblasts, hepatocytes, inhibition of B and T lymphocytes and natural killer cells (NK cells) and decrease of production of antibodies. It also inhibits "the plasminogen activator", stromelysin and collagenase. Under physiological conditions, thrombocytes serve as sources of TGF-β. After the stimulation, further cell elements participate in TGF-β production, prevalently fibroblasts, myofibroblasts and eosinophilic leukocytes, activated monocytes and epidermal cells (Randall and Coggle, 1996; Grande et al., 1997; Wang et al., 1998).

TGF-β belongs into the family of polypeptidic factors controlling the development and tissue homeostasis in all the animal species. In mammals, isoforms TGF-β 1, 2 and 3 are expressed; their activities are overlapping to a considerable extent. The activity of TGF-β1 was studied particularly in relation to the production of the extracellular matrix (Grande, 1997).

The TGF-β1 expression was experimentally demonstrated in many models of advanced secondary fibrosing processes, including the postirradiation reactions (Randall and Coggle, 1995; Hauer-Jensen et al., 1998). The TGF-β1 serum level is considered as an indicator of progression of the fibrosing process (Vujaskovic et al., 1997; Anscher et al., 1998). The function of TGF-β1 has been relatively well defined in early as well as in advanced stages of postirradiation pneumopathy (Li et al., 1999). However, mechanisms remain non-documented that start the whole process and that lead with a certain latency to the pathological fibroproduction (Herskind et al., 1998). From this standpoint, the effect of growth factors from thrombocyte granules as a starter of the tissue TGF-β1 autocrine production loop

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Abbreviations: IL – interleukin, RT-PCR – reverse-trancscriptase polymerase chain reaction, TGF-β – transforming growth factor β, TNF-α – tumour-necrosis factor α.

is of interest. In the experimental bleomycin pneumopathy it was demonstrated that after i.v. injection of bleomycin, a considerable increase of thrombocytes aggregated to endothelial cells of capillaries occurred in the lung parenchyma after three weeks, particularly in areas of collagenous deposits (Piguet and Vesin, 1994). A decrease of thrombocyte aggregation was achieved using antibodies against CD11a, CD11b, TNF-α and IL-1R (Tacchini-Cottier et al., 1998).

The radiation as well as bleomycin potential to induce lung fibrosis is affected by a common genetic factor (Li at al., 1996; Haston and Travis, 1997. In addition, the enhanced aggregation of thrombocytes to the endothelium is a universal reaction to the tissue damage (Senaldi and Piguet, 1997). In the present work we studied TGF-β1 mRNA production in the lung tissue of mice after their whole-body low-dose irradiation. Thrombocyte trapping in the lung vessels was followed up, since it is known that the number of thrombocytes in peripheral blood rapidly decreases in the acute postirradiation stage. In the case of a persisting enhanced "trapping" in the acute postirradiation stage, the mechanism of thrombocytopenia would be a generalized aggregation of platelets in organs. During its protracted existence it could become the factor associating platelet growth factors and TGF-β1 tissue production with closing the *circulus vitiosus* between extra- and intravascular factors of the repair.

Two mouse strains were used for the experimental comparison (C3H/J and C57BL/6), which exerted different tendencies to develop processes of fibrosing (Franko et al., 1997). The radiation dose used in the presented experiment was well below those shown to induce fibrosis in the fibrosing C57BL/6 strain. However, the aim of the study was not to develop the fibrosing process in both mice strains, but to follow up acute and subacute discrete cytokine production after a low-dose whole-body radiation stimulus, known to induce thrombocytopenia.

Material and Methods

Animals and radiation

Inbred C3H/J and C57BL/6 strain female mice of body weights of 18 to 20 g (AnLab Ltd. Charles River, Czech Republic) were used in the experiment. The mice were kept in a barrier facility for animals, provided with radiation-sterilized bedding (SAWI Research Bedding, Jelu-Werk, Rosenberg, Germany), fed with the radiation-sterilized ST-1 diet (Bergmann-Kocanda, Jesenice, Czech Republic) and they received autoclaved water *ad libitum*.

The mice of both strains were exposed to a whole-body dose of 7 Gy (^{60}Co was used; radiation distance 121.5 cm; radiation input 0.3248 Gy/min). The total of 60 animals was included in the experiments. Ten animals of each strain were sacrificed on day 9 after irradiation and 10 animals of each strain were also sacrificed 8 weeks after irradiation. Ten mice of each group were used as controls. Peripheral blood samples from all the animals were centrifuged and the blood serum was obtained. Blood elements were simultaneously counted. The animals were also subjected to evisceration and the lungs were divided *ana partes equales* and deeply frozen for reverse-transcriptase polymerase chain reaction (RT-PCR) examination of TGF-β1 or fixed with glutaraldehyde for electron-optical examination. The number of megakaryocytes in bone marrow was also examined.

RT-PCR

Lung samples (20 mg) were homogenized and incubated with 1 ml of the RNA Blue (Top-Bio, Prague, Czech Republic). After homogenization, 0.2 ml of chloroform was added and after short incubation at room temperature (RT) (3 min) the samples were centrifuged at 12.000 × *g* for 15 min at 4°C. The aqueous phase was removed into a new eppendorf tube, 0.5 isopropanol was added, vortexed and incubated for 10 min at RT. Samples were centrifuged at 12.000 × *g* for 10 min at 4°C. Supernatant was discarded and the sediment vortexed with 1 ml of 75% ethanol and centrifuged for 5 min at 12.000 × *g*, 4°C. After washing with 80% ethanol, the pellet was dried in vacuum and finally dissolved in 20 μl of diethyl pyrocarbonate (DEPC) water. Concentration of RNA was measured using a spectrophotometer at 280/260 nm. Equally, 1 μg of RNA was used per each reverse-transcriptase reaction. Primers for TGF-β1 were from Clontech, Palo Alto, CA. β-actin served as an internal control. Other components for RT-PCR were from the RT-PCR kit obtained from Top-Bio, Prague, Czech Republic. RT-PCR was done according to the supplier's instructions. PCR was run in a PTC 200 cycler.

Products were analyzed eletrophoretically on a 1.5% agarose gel with ethidium bromide. The picture of the gel was taken with computered camera using GRAB-IT software and analyzed with the GEL-BASE software. For results, the main peak of each line was measured and compared with the control. The results are semiquantitative.

Electron microscopy

Two 1-mm^2 lung blocks from different regions per each mouse were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 12 h at ambient temperature. After being rinsed three times for 20 min in the same buffer, the material was postfixed for 6 h in 1% osmium tetroxide (in 0.1 M sodium cacodylate buffer), dehydrated in acetone and in Epon 812 (Fluka, Buchs, Switzerland). Semithin sections were cut with a Leica Ultracut S Microtom. Ultrathin sections were prepared, stained with uranyl acetate and examined in a Tesla electron microscope (Tesla, Brno, Czech Republic). The ultrathin sections were scanned systematically under