Redox-Mediated Activation of Latent Transforming Growth Factor-β1

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Transforming growth factor β1 (TGFβ) is a multifunctional cytokine that orchestrates response to injury via ubiquitous cell surface receptors. The biological activity of TGFβ is restrained by its secretion as a latent complex (LTGFP) such that activation determines the extent of TGFβ activity during physiological and pathological events. TGFβ action has been implicated in a variety of reactive oxygen-mediated tissue processes, particularly inflammation, and in pathologies such as repertusion injury, rheumatoid arthritis, and atherosclerosis. It was recently shown to be rapidly activated after in vivo radiation exposure, which also generates reactive oxygen species (ROS). In the present studies, the potential for redox-mediated LTGFP activation was investigated using a cell-free system in which ROS were generated in solution by ionizing radiation or metal ion-catalyzed ascorbate reaction. Irradiation (100 Gray) of recombinant human LTGFP in solution induced 26% activation compared with that elicited by standard thermal activation. Metal-catalyzed ascorbate oxidation elicited extremely efficient recombinant LTGFP activation that matched or exceeded thermal activation. The efficiency of ascorbate activation depended on ascorbate concentration and the presence of transition metal ions. We postulate that oxidation of specific amino acids in the latency-conferring peptide leads to a conformation change in the latent complex that allows release of TGFβ.

Oxidative activation offers a novel route for the involvement of TGFβ in tissue processes in which ROS are implicated and endows LTGFP with the ability to act as a sensor of oxidative stress and, by releasing TGFβ, to function as a signal for orchestrating the response of multiple cell types. LTGFP redox sensitivity is presumably directed toward recovery of homeostasis; however, oxidation may also be a mechanism of LTGFP activation that can be deleterious during disease mechanisms involving chronic ROS production. (Molecular Endocrinology 10: 1077–1083, 1996)

INTRODUCTION

Transforming growth factor-β1 (TGFβ) is a multifunctional cytokine that orchestrates cell type-specific regulation of cell cycle, growth, differentiation, and death via ubiquitous cell surface receptors. Its activity is controlled by secretion as a latent complex (LTGFP), which is widely distributed in tissues (1). Latency is conferred by a noncovalent association of TGFβ with the latency-associated peptide (LAP), which is the amino-terminal portion of the TGFβ precursor processed into an 80-kDa homodimer (2). Activation consists of releasing TGFβ from LAP, which occurs after heat or acid treatment (3), plasmin proteolysis (4–6), deglycosylation (9), or binding to thrombospondin (10). Activation is considered to be the critical regulatory control for the biological action of TGFβ (11). However, the mechanisms of LTGFP activation employed in vivo under physiological stress are not well understood. Furthermore, while LTGFP activation is an important switch to initiate tissue response to injury, it can be deleterious when events lead to overproduction of TGFβ (12, 13). Two pivotal questions regarding the role of TGFβ in vivo are: when and how does LTGFP become activated?

We recently demonstrated immunohistochemical evidence suggestive of LTGFP activation in mouse mammary gland within 1 h of ionizing radiation exposure in vivo (14). Two significant alterations in immunoreactivity were noted: increased access to certain N-terminus epitopes of mature TGFβ and decreased stability of, or access to, LAP (14). TGFβ immunoreactivity is detected within 1 h of radiation exposure, persists for 7 days post irradiation, and inversely correlates with LAP immunodetection over a similar time course. We postulated that these radiation-induced changes in immunoreactivity represent the first localization of activation in situ.
At 24 h, collagen type III, a known target of TGFβ, is expressed in the adipose stroma in a pattern that colocalizes with TGFβ induction (15). Together with the known actions of TGFβ on extracellular matrix production (16–18), the temporal relationship between TGFβ induction and the subsequent colocalization with collagen III suggests the hypothesis that radiation induces LTGFβ activation, which then mediates remodeling of the stromal extracellular matrix. TGFβ and collagen III also show similar graded induction with increasing radiation doses from 0.5 to 5 Gray (Gy). This correlation was confirmed by showing that TGFβ neutralizing antibodies administered to mice before irradiation block radiation-induced collagen III (E.J. Ehrhar, P. Segarini, M.E.S. Tsang, and M. H. Barcellos-Hoff, submitted). Our interpretation of the differential immunolocalization of active and latent TGFβ suggests that ionizing radiation rapidly induces TGFβ activation.

A major mode of ionizing radiation action is the generation of hydroxyl radicals and other reactive oxygen species (ROS) (19). We reasoned that the rapidity of TGFβ activation in the irradiated mammary gland suggested an effect attributable to ROS exposure. LTGFβ activation also appears to be concomitant with ROS generation by leukocytes (20, 21) and has been implicated in a number of diseases in which production of ROS contributes to the disease state or progression (22–30). We postulated that LTGFβ activation is similar to the redox sensitivity exhibited by certain transcriptional factors (31). This hypothesis was studied in a cell-free model in which recombinant human LTGFβ was exposed to various ROS-generating mechanisms. TGFβ release was measured using a mink lung epithelial cell biological assay to determine the degree of activation under these experimental conditions as compared with standard thermal activation. We observed highly efficient activation as a consequence of exposure to ROS generated by metal ion-catalyzed ascorbate oxidation and Fenton chemistry, with little deleterious effect on the activity of TGFβ itself. We propose that LTGFβ is susceptible to redox-mediated alterations in conformation or stability that result in activation and discuss the potential impact of this mechanism on understanding tissue processes in which TGFβ is implicated.

RESULTS

Irradiating LTGFβ in Solution Elicits Activation

Based on the rapid (<1 h) induction of LTGFβ activation in mouse mammary gland irradiated in vivo (14), we tested the hypothesis that LTGFβ is activated by the reactive oxygen products generated by the indirect action of ionizing radiation. Ionizing radiation produces highly reactive hydroxyl radicals and other ROS in aqueous solution that randomly react with targets in free solution at near diffusion-controlled rates. Re-combinant native human LTGFβ (rLTGFβ) was irradiated with 50–200 Gy of 60Co-γ radiation in iron-containing saline. Irradiation resulted in a significant increase of TGFβ activity as determined by induction of PAI-luciferase biological activity (Table 1). Irradiation in solution with 100 Gy resulted in 26% activation compared with that elicited by thermal activation of untreated LTGFβ. However, since thermal activation of irradiated LTGFβ decreased with increasing radiation exposure, these doses of radiation appear to both activate and damage LTGFβ. When degradation is taken into account by comparing the activity of LTGFβ irradiated with 100 Gy to its own thermal control, 41% of the potentially activatable TGFβ was elicited by radiation alone. Activation by ionizing radiation compares favorably with activation mediated by proteases such as plasmin, which activates 15–20% of rLTGFβ in the presence of high levels of enzymatic activity and also degrades TGFβ (3).

Our previous observations in irradiated mammary gland detected activation immunohistochemically with doses of 5 Gy and less (14). The relative inefficiency of activation in terms of dose using solution irradiation of rLTGFβ may reflect the relative sensitivity of different endpoints (i.e. immunohistochemistry vs. biological activity), the presence of biological mediators in vivo, or differences in quantity or composition of the ROS generated in solution compared with those generated by ionizations in vivo. The initial deposition of energy by radiation in biological systems is random, with about 25% of the energy being absorbed directly by matter and 75% of the energy absorbed by water, which results in production of ROS, specifically hydroxyl radical, via radiolysis of water. We postulate that the latter mode of action prevails in the activation of LTGFβ, while the former is detrimental. Furthermore, some mechanisms of radical propagation (e.g. lipid peroxidation in membranes) are only tenable in biological systems. Thus, secondary reactions generating other ROS species in the local environment may also be critical to biological effects in vivo (19).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active TGFβ</th>
<th>Thermal Activation</th>
<th>% Thermal Activation</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.7 ± 1.1</td>
<td>119.8 ± 16.6</td>
<td>1.4</td>
</tr>
<tr>
<td>50 Gy</td>
<td>17.6 ± 3.8</td>
<td>65.6 ± 21.6</td>
<td>26</td>
</tr>
<tr>
<td>100 Gy</td>
<td>30.8 ± 14</td>
<td>75 ± 40</td>
<td>41</td>
</tr>
<tr>
<td>200 Gy</td>
<td>16.8 ± 10.7</td>
<td>32.8 ± 12.8</td>
<td>51</td>
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</table>

MLE-PAIL cultures were incubated with control rLTGFβ or LTGFβ irradiated with 60Co-γ radiation in metal-depleted saline diluent. Thermal activation serves as a control for total activation. The amount of biologically active TGFβ present in each sample was determined by extrapolation from the standard curve. The units are picograms and the values are mean ± sd of triplicate determinations.
Metal Ion-Catalyzed Ascorbate Oxidation Elicits Efficient Activation

We tested the effect of hydrogen peroxide, a relatively persistent product of ionizing radiation (19), on latent and active TGFβ. Recombinant LTGFβ or recombinant human TGFβ (rTGFβ) was exposed to concentrations of 10–400 μM hydrogen peroxide for 10 min in saline diluent, but neither LTGFβ activation nor TGFβ activity was affected (data not shown). To generate a spectrum of ROS that approximates the heterogeneity of events in vivo, we used ascorbate oxidation catalyzed by the addition of 20 μM Fe³⁺/EDTA or Cu²⁺. This reactive oxidant-generating system produces hydroxyl radicals, superoxide ions, and hydrogen peroxide (32). Compared with thermal activation in each diluent, ascorbate-induced activation was 26% in copper-containing saline, 179% in iron-containing saline, and 217% in metal-depleted saline. These data indicate that ROS-induced activation exceeds thermal activation of rTGFβ under certain conditions.

Transition Metals Affect Both Thermal and Ascorbate-Induced LTGFβ Activation

Surprisingly, the efficiency of thermal activation was found to depend on the metal composition of the saline diluent (Table 2). The addition of iron or copper significantly increased thermal activation compared with that obtained in metal-depleted saline. As a consequence, ascorbate-induced activation in the presence of copper was less efficient than thermal activation. In addition, endogenous TGFβ activity detected after incubation in the metal-containing diluents increased as compared with that from rLTGFβ incubated in metal-depleted saline, although the percent activation of the untreated LTGFβ was not affected. This suggested that perhaps the stability of active TGFβ was affected by the presence of transition metal ions, which could lead to greater recovery after thermal activation. This possibility was tested by incubating rTGFβ in each saline diluent for the same duration as the ascorbate treatment. However, rTGFβ biological activity, with or without thermal treatment, was actually slightly inhibited in iron-containing diluent compared with metal-depleted saline. Thus, the change in thermal activation was not due to increased biological stability of TGFβ in the presence of metal ions (Table 3). Importantly, exposure to ascorbate in iron- or metal-depleted saline diluent had little deleterious effect on rTGFβ biological activity. Therefore, exposure to ROS per se did not inhibit the biological activity of TGFβ.

Ascorbate-induced activation showed a dose response at concentrations of less than 500 μg/ml in all saline diluents (Fig. 1). Activation was inhibited at the highest concentration (1 mg/ml), suggesting that ascorbate’s antioxidant effects may compete with prooxidant action when metal-ion concentration is limited. At the lowest ascorbate concentration (125 μg/ml) there was no activation in metal-depleted saline. The inhibition of ascorbate-induced activation in metal-depleted saline at low ascorbate concentrations compared with saline containing either iron or copper is consistent with the dependence of ascorbate’s prooxidant effect on transition metal catalysts.

ROS-Induced Activation Persists in the Presence of Metal Chelators

The observation that activation was not completely inhibited by metal depletion suggested that either metal depletion was incomplete or that there are other sources of transition metal available to catalyze the ascorbate oxidation. Therefore, we tested whether the addition of transition metal chelators would block activation in metal-depleted saline. Ascorbate activation of rTGFβ in metal-depleted saline was similar to the ascorbate treatment alone when pretreated for 1 h with either 100 μM diethylenetriamine pentaacetic acid (98%), a chelator of both iron and copper, or 400 μM desferoxamine (92%), a strong inhibitor of iron-redox cycling, before incubation with ascorbate. As these chelators did not affect the efficiency of activation by ascorbate in metal-depleted saline, these data are consistent with the possibility that metal ions were inaccessible to chelation, which has been observed for

<table>
<thead>
<tr>
<th>Table 2. Ascorbate-Generated ROS Activates LTGF-β as a Function of Metal Ion</th>
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<tr>
<td>rLTGF-β Treatment</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Heat; 80 C, 5 min</td>
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<tr>
<td>ROS; ascorbate, 500 μg/ml, 2 h</td>
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MLE-PAIL cultures were treated with rLTGF-β following control, thermal, or reactive oxygen exposure in each saline diluent. The amount of biologically active TGFβ present in each sample was determined by extrapolation from the standard curve. The units are picograms and the values are the mean ± SE for the indicated number (n) of experiments. The percent of active TGFβ released by ROS was calculated using heat activation in each diluent as the respective standard.
### Table 3. rTGF-β Activity as a Function of Metal Ions ± Ascorbate

<table>
<thead>
<tr>
<th>rTGF-β Treatment</th>
<th>Metal-Depleted Saline</th>
<th>Fe³⁺/EDTA Saline</th>
<th>Cu⁺⁺-Containing Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>163.4 ± 29.5</td>
<td>103.5 ± 10.3</td>
<td>173 ± 86.9</td>
</tr>
<tr>
<td>Ascorbate (500 μg/ml)</td>
<td>278.3 ± 17.4</td>
<td>170.1 ± 64.2</td>
<td>57.2 ± 21.3</td>
</tr>
</tbody>
</table>

MLE-PAIL cultures were treated with rTGF-β following control or reactive oxygen exposure (2 hr) in each saline diluent. The amount of biologically active TGF-β present in each sample was determined by extrapolation from the standard curve. The units are picograms and the values are the mean ± standard deviation of triplicate determination.

Several proteins that specifically bind transition metals (33).

**DISCUSSION**

Efficient activation of the LTGFβ was achieved in a cell-free system using two standard sources of ROS, ionizing radiation and metal ion-catalyzed ascorbate oxidation, which offers new insights into the potential regulation of this critical event in vivo. Each of these ROS sources model physiological processes in which high localized concentrations of oxidants are generated, supporting the hypothesis that LTGFβ activation may be redox sensitive in vivo under certain conditions. This hypothesis was suggested by our studies that revealed immunohistochemical evidence of LTGFβ activation within 1 h of irradiation in the tissue microenvironment of the mouse mammary gland in vivo (14). Previous observations supporting a potential role of ROS in LTGFβ activation are the known involvement of LTGFβ in inflammation (20, 21) and its implication in a variety of disease processes, including acute respiratory distress syndrome (22), reperfusion injury (23, 24), cerebral trauma or ischemia (25, 26), atherosclerosis (27–29), and arthritis (30), in which ROS generation is a potential etiological mechanism.

Activation of rLTGFβ by both irradiation and ROS generated by ascorbate in the presence of metal catalysts proved to be very efficient in this cell-free system. While radiation exposure was deleterious to subsequent LTGFβ thermal activation, ascorbate-generated ROS did not inhibit the activity of rLTGFβ itself. Protease-mediated activation of latent LTGFβ is generally considered to be the primary means of releasing biologically active LTGFβ from the latent complex. The mechanism of LTGFβ activation upon exposure to ROS compares quite favorably with plasmin activation, in terms of both efficiency and specificity. Plasmin-dependent activation generates from 5–20% activation concomitant with an inhibitory effect due to degradation of LTGFβ (4). Oxidation-mediated activation offers a novel route for LTGFβ activity in chronic tissue processes in which oxidative stress is implicated. ROS have been postulated to play a role in extra- and intracellular signaling (34). LTGFβ could sense extracellular ROS and also rapidly transduce the signal to elicit changes in phenotype. If this mechanism of activation occurs in vivo, then oxidative stress could result in LTGFβ activation.

The presence and type of transition metal catalysts affected the efficiency of ascorbate-induced activation, as is consistent with ascorbate prooxidant chemistry (33). We found that a solution source of H₂O₂ was unable to augment the extent of ascorbate activation (data not shown), which suggests that O₂ is the oxidant that is being activated. The ability of iron-ascorbate or copper-ascorbate complexes to activate O₂ is well documented (33). Surprisingly, thermal activation was also significantly influenced by the metal composition of the reaction by an as yet unknown mechanism, suggesting that caution should be exercised in evaluating activation in different chemical environments.

The data demonstrate that the oxidative events occur specifically in the latent complex, rather than by affecting the active LTGFβ polypeptide, which suggests...
that redox susceptibility resides in the LAP molecule. LAP contains numerous amino acids with redox potential. Three cysteine positions have been shown by site-directed mutation to result in constitutive LTGFB activation when replaced with serine (35). LAP also contains 10 methionines, which are frequently the site of specific oxidative protein inactivation (36). Thus, there are several sites at which oxidation may alter LAP protein conformation or stability.

Since TGFB isoforms show only 30-45% sequence identity in the region of the gene encoding LAP, susceptibility to redox activation could be isoform specific. The addition of exogenous metal ion catalysts increased the efficiency of activation; however, ascorbate activation was not completely inhibited by metal depletion even in the presence of chelators. Highly reactive radicals, such as the hydroxyl radical, recombine at, or very near, the site of formation, which, in the case of ascorbate, would be close to the location of catalytic metal ions. Unlike ionizing radiation, which generates free radicals randomly in relation to a given target protein, metal ion-catalyzed ascorbate reactions can be site-specific if the protein binds a transition metal ion (32). We propose the following hypothesis: ascorbate reacts with a transition metal bound either adventitiously or specifically to LAP, which leads to site-specific oxidation of certain amino acids in LAP. We postulate that oxidation of amino acids leads to a conformational change in LAP that allows TGFB to be released from the latent complex. Recent structural studies demonstrate that LAP undergoes conformational changes when associated with TGFB (37). Further support for this hypothesis will evolve from structural studies of LAP that include the evaluation of protein-bound transition metals and site-directed mutagenesis of potentially oxidizable amino acid residues.

ROS-dependent activation of LTGFB would be fast, diffuse, and protein-synthesis independent, as might be required in tissue response to damage or infection. Although ROS-induced activation would be less well controlled compared with cell surface plasmin-mediated activation as described by Rifkin and others (5-8), it would have features suitable to its role in orchestrating the rapid response of tissue to damage. ROS-mediated release of extracellular TGFB would expose cells that lack the proteolytic machinery for activation and thus recruit them into the damage response, much as platelet degranulation recruits appropriate cells to the site of a wound. Activation distant from cell surface receptors would favor TGFB’s role as a chemoattractant at sites of inflammation (38). Macrophages, monocytes, and polymorphonuclear leukocytes activate LTGFB after stimulation (20, 21) and are a potential cellular source for ROS. During wound healing, cell-generated ROS might perpetuate activation of extracellular stores of LTGFB, which could then serve as a gradient for chemotaxis and a stimulus for fibroblast and monocyte phenotypic conversion. As such, ROS-mediated activation would endow LTGFB with the ability to act as a sensor of oxidative stress and, in releasing TGFB, to signal multiple cell types to change their phenotype, presumably directed toward recovery of homeostasis. However, TGFB has also been implicated in a variety of ROS-mediated tissue pathologies that include acute respiratory distress syndrome (22), atherosclerosis (29), and radiogenic fibrosis (39, 40), which suggests that oxidative activation of LTGFB may be deleterious to tissue in certain disease states leading to chronic production of ROS.

Interestingly, there is mounting evidence that TGFB itself may signal certain events through the generation of ROS (41-46). TGFB induces the production of hydrogen peroxide in bovine endothelial cells (47), mouse osteoblastic cells, where it has been shown to be necessary for the transcriptional activation of the egr-1 gene (43), and human lung fibroblasts, where it is generated by the activation of NADH oxidase (44). Das and Fanburg (48) have suggested that TGFB in the presence of FeCl3 leads to a prooxidant state in bovine endothelial cells, in part through regulation of Cu and Zn-superoxide dismutase (49) and depletion of glutathione (50). Similar suppression of antioxidative enzyme expression is found in rat hepatocytes (51). Recent studies have implicated ROS as an important signal for TGFB-induced apoptosis (45, 46). A variety of tumor cells generate ROS (52), which could contribute to nonspecific LTGFB activation leading to immunosuppression or stromal reaction during carcinogenesis (53). Thus, in some situations, a self-amplifying cascade may be envisioned in which TGFB stimulates nonphagocytic cells to produce ROS, which then contribute to further activation via the redox sensitivity of the LTGFB.

**MATERIALS AND METHODS**

Recombinant native human LTGFB, which is the 100- to 110-kDa small latent complex, was provided by Dr. Monica Tsao (R & D Systems, Minneapolis, MN). "Native" LTGFB purchased from R & D Systems was used as the standard for bioassays and treated in parallel experiments. All chemicals were reagent grade and were purchased from Sigma Pharmaceuticals (St. Louis, MO) unless otherwise noted. All experiments were conducted in siliconized, plastic tubes, and solutions were prepared with sterile, deionized water. rLTGFB or rTGFp was incubated at 37 °C with experimental constituents for 2 h with agitation in a total volume of 40-60 μl and then diluted 100- to 1000-fold to bring the sample concentration into the effective range of the bioassay and to minimize the potential for reassociation of the latent complex (3). rLTGFB was exposed to ascorbate in saline (0.9% NaCl), prepared with deionized water, and depleted of metals by stirring with Chelex-100 resin (1 g/10 ml, Pierce, Rockford, IL) overnight. Ferric chloride (20 μM plus 200 μM EDTA) or cupric chloride (20 μM) was added to metal-depleted saline immediately before addition of protein in some experiments. Experimental constituents were tested for bioactivity by performing the incubation without addition of the cytokine.

TGFB activity was determined using a bioassay consisting of mink lung epithelial cells transfected with a TGFB-responsive plasminogen activator inhibitor-1 (PAI) promoter-luciferase construct (54). Luciferase activity is proportional to rTGFB concentration and was assayed with the following...
minor modifications: 1.6 x 10³ transfected cells were plated in 96-well plates in 50 µl of 0.5% FBS in DMEM culture media at 100-800 pg/ml rTGFβ in each sample diluted in 0.2% serum containing DMEM were added and incubated for an additional 16 h. Medium was removed from the plates, which were washed twice with PBS, before the addition of lysis buffer (50 µl) consisting of 25 mM Tris, 10 mM 1,2-diaminocyclohexane-tetraacetic acid, 2 mM dithiothreitol, 1% glycerol, and 1% Triton X-100. Forty microliters of each sample were transferred to an opaque luminometer 96-well plate and luciferase substrate, consisting of 20 mM tricine, 1.07 mM (MgCO₃), MgOH₃, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 µM coenzyme-A, 470 µM luciferin (Promega Corp., Madison, WI) and 530 µM ATP, pH 7.8, was injected by an automated EG & G Berthold Micolumat LB 96P (Oak Ridge, TN) luminometer. Luciferase activity was measured immediately for 10 sec and recorded as relative light units and used to determine the amount. Total rTGFβ activation was determined by thermal activation (5 min at 80 C) (3).

Acknowledgments

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Oxidative Activation of Latent TGFβ


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