EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 35, No. 6, 565-571, December 2003

# Role of crosslinked protein in lung injury following total body irradiation and bone marrow transplantation

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Accepted 8 December 2003

Abbreviations: BAL, bronchoalveolar larvage; BMT, bone marrow transplantation; DTT, dithiothreitol; IP, interstitial pneumonitis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; SGM, standard gas mixture; TBI, total body irradiation; TBS, tris-buffered saline; TGase, transglutaminase; XIII, blood coagulation factor XIII; VCO, carbon monoxide uptake

## Abstract

**The aberrant protein crosslinks formation during lung injury as results total body irradiation (TBI) and bone marrow transplantation (BMT) therapy has been examined as apossible contributory factor in organ or tissue pathogenesis. Female C3HeB/FeJ mice were used for an experimental animal. Carbon monoxide uptake ($V_{CO}$) was measured at 1, 2, 3, 4 and 5 months after TBI at respective doses of 12, 14, 16 and 18 Gy 16 h prior to syngeneic BMT. Also as a measure of aberrant protein crosslinking in the inured tissues, transglutaminase (TGase)-activities and crosslinked protein were examined along with thrombin, a protease known to activate TGases. Reductions of $V_{CO}$ were detected following TBI and BMT. Activities of thrombin and TGase 1, and crosslinked protein in bronchoalveolar lavage (BAL) fluid of the mice 1 wk after TBI at 12 Gy and BMT were identified and found to be elevated in the treated animals. These findings suggest that elevated levels of crosslinked proteins and TGase I in the bronchoalveolar larvage during the lung injury could have enhanced the organ pathogenesis following TBI and BMT.**

**Keywords:** carbon monoxide uptake; crosslinked protein; thrombin; total body irradiation and bone marrow transplantation; transglutaminases

## Introduction

Bone marrow transplant (BMT) has become an effective treatment choice for various hemopoietic disorders such as acute leukemias, immune deficiency disorders, congenital metabolic disorders and severe aplastic anemia (Kersey *et al.*, 1982; McGlave *et al.*, 1982; O'Leary *et al.*, 1983; Rappaport *et al.*, 1983; Molls *et al.*, 1986; Vriesendorp *et al.*, 1994). The effectiveness of BMT is crossly associated with patient status of prior elimination of existing malignant plasma cells by excessive irradiation treatment. During irradiation therapy, some organs are more sensitive than other tissues where lung is one of the organs known to show a dose-limiting tolerance for total body irradiation (TBI). The frequent failure of BMT therapy may be due to an incidence of interstitial pneumonitis (IP). The reported incidence of IP in allogeneic BMT ranged from 29% to 55% with a fatality rate of up to 69% (Penny and Rubin, 1997). It is estimated that about 25% of patients who undergo allogeneic BMT die from conditions related to IP. The etiology of IP is not clearly known. However, the effect of radiation on lung tissue is considered to be one of the major contributing factors. Recently, many candidates for inducing lung radiation injury have been reported-TGF-β (Anscher *et al.*, 1995), IL-4 (Büttner *et al.*, 1997), IL-6 (Chen *et al.*, 2001) and thrombin (Huang *et al.*, 2001). Aberrant protein oxidation may have induced irreversible aggregation and protein cross-linking in the inflamed and injured tissue due to excess irradiation. Such radiation caused protein crosslinking may have precipitated consequential tissue fibriosis. Protein crosslinking is catalyzed by transglutaminases (TGases) that are known to catalyze $N^{\varepsilon}$-(γ-glutamyl) lysine crosslinks. Multiple molecular forms of TGase have been identified to date and have been found to exhibit differences in specificity. Among them, TGase 1, 3 and 4 were shown to be activated by proteolytic processing into forms with high specific activities respectively. One of the proteolytic enzymes was shown to be thrombin (Chung *et al.*, 1998). The irreversible nature of fibrosis could have been contributed by the formation of covalent intermolecular crosslinks between different proteins by $Ca^{2+}$-dependent TGase (Richards *et al.*, 1991).

To date, the nature of radiation-induced early events that lead to the pathogenesis of the late lung injury remains obscure. In order to explore possible contribution of aberrant protein aggregation and crosslinking in eliciting pathogenesis of radiation-induced lung injury during TBI and BMT treatment, carbon monoxide uptake ($V_{CO}$) was measured following TBI and BMT and the levels of thrombin, TGases and crosslinked protein were analyzed in bronchoalveolar larvage (BAL) fluid during the early stages of TBI and BMT.

## Materials and Methods

### Animals and animal care

Female C3HeB/FeJ mice at 6-8 wks of age were obtained from Jackson Laboratory. They received neomycin-terramycin (Pfizer Co.), 0.3 g/L tetracycline HCl and 0.2 g/L neomycin sulfate triweekly in their drinking water. Both irradiated and unirradiated control animals were maintained on antibiotics throughout the experiment. In addition, mice receiving TBI and their unirradiated controls were housed in sterilized cages with filter bonnets, fed with autoclaved Purina Lab Chow (Samyang Co. Wonju, Korea), and given sterilized water for a period of 8 wks. The use of antibiotics and sterile conditions has been shown to improve survival and prevent respiratory infection following TBI. Animals were maintained in a temperature-controlled environment at 22°C on a 12 h light/dark cycle.

### Irradiation and dosimetry

TBIs were carried out using a $^{60}Co$ source. Mice were caged in lucite chambers 3 cm deep, 3 cm wide, and 6 cm long with a 0.5 cm thick lucite cover containing holes for ventilation. Eighty four such chambers were contained within a single irradiation jig. The chambers were sterilized prior to irradiation by immersion in 70% ethanol. Mice remained unanesthetized during irradiation and breathed room air. Dose rate to the mouse midline was measured in a polystyrene phantom at a depth of 1.2 cm using a calibrated ionization chamber. Maximum buildup of dose was achieved at the surface of the mouse jig. Uniformity of dose across the field was established by placing a piece of radiographic verification film between two 1 cm thick polystyrene sheets at the position of the mouse midline. Polystyrene sheets were placed around the mouse jig and rice bags were placed in chambers not containing mice to insure uniformity of dose due to scattered radiation. The animals received TBI; 12 Gy, 14 Gy, 16 Gy and 18 Gy, at dose rate of 2.5 cGy/min.

### BMT

Animals receiving TBI also received a syngeneic bone marrow transplantation at 16-24 h following irradiation. Bone marrow was collected and prepared for transplantation by the method of Vallera *et al.* (1981). Approximately $2\times10^6$ nucleated bone marrow cells were inoculated intravenously into the tail vein of each recipient mouse.

### Steady-state $V_{CO}$

$V_{CO}$ was determined according to the technique of DePledge *et al.* (1981). The concentration of rebreathed carbon monoxide (CO) and helium (He) were determined by CO and He analyzers (Hewlett-Packard He analyzer No. 47313A, CO analyzer No. 47312A, Willmington, Delaware). Output from the gas analyzers was read on a digital display or from a strip chart recorder. The standard gas mixture (SGM) used contained 10% He, 0.3% CO, 21% $O_2$ and balanced $N_2$ (Matheson Gas Products, Joliet, IL).

Mice were placed into a 100 ml syringe, and the plunger was inserted to confine the mouse in a minimum volume without compression. Approximately 80 ml of SGM was drawn via a gas collection bag into the 100 ml syringe. The mice were allowed to rebreathe the gas for 90 s, during which time the gas collection bag was flushed with air and evacuated. At the end of the rebreathing period, gas was expelled into the evacuated collection bag and then automatically drawn into the analyzers. The percentage concentration of CO at the beginning and conclusion of the rebreathing period ($V_{CO}$) was calculated according to DePledge *et al.* (1981). Before each experiment the gas analyzers were calibrated with 80 ml samples of SGM or 80 ml samples of gas containing various proportions of SGM and air. This procedure was performed to determine factors ($CF_{He}$ and $CF_{CO}$) used to correct for sample size and flow dependence. In order to minimize the possible effects of diurnal rhythm, determinations of $V_{CO}$ were made during the early afternoon.

### BAL

After trachea cannulation with plastic catheters attached to a 1 ml syringe, the lungs were washed with sterile saline in five 0.6 ml portions by gentle massage, and BAL fluid was collected to a total volume of 3 ml. The BAL fluid was centrifuged at 1,200 rpm for 10 min at 4°C to precipitate the cells. The supernatant was stored at -80°C before assay for thrombin and TGase activities, and identification of crosslinked protein.

### Determination of protease activity of thrombin in BAL fluid

The protease activity of thrombin was assayed fluorometrically (16). Briefly, 80 μl of 0.1 M Tris-HCl buffer (pH 8.0), 100 μl of 100 μM Boc-Val-Pro-Arg-MCA diluted with the buffer, and 20 μl BAL fluid were added to the wells of 96 well plates (8 White Maxisorp, Nunc, Denmark). The standard buffer contained various amounts of 7-amino-4-methylcoumarin. The plates were incubated at 37°C for 1 h. Fluorescence intensity was measured by an MTP-32 (Hitachi, Tokyo, Japan) with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Thrombin activity was expressed in nanomoles of substrate cleaved per h.

### TGase and XIII activity assay

The thrombin activated FXIII- and TGase-activities were measured by an incorporation of [1,4$^{14}$C] putrescine (100 μCi/mole) into the succinylated Hammersten casein assay as described (8). The assay solution contained 0.1 M Tris-acetate buffer pH 8.0, 1 mM EDTA, 10 mM $CaCl_2$, 5 mM DTT, 0.5% Lubrol (Sigma) and 1% succinylated Hammersten casein. The reaction was started with an addition of 50 μl of 0.5 Ci radioactive putrescine into the 0.45 ml of assay mixture containing 50 μl of BAL fluid, and 1 U of thrombin at 37°C and stopped by addition of 4 ml 10% cold TCA solution. The casein precipitate was filtered on the glass filter (Whatmann GF/A) and the radioactivity was measured.

Distributions of TGase 1 and TGase 2 were examined by immunohistochemistry. For immunohistochemistry, 5-μm sections were cut from wax-embedded tissue and immunostaining was detected by Tyramide Signal Amplification-Indirect Immunohistochemistry kit (NEN, Boston, MA). Sections were rehydrated and endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide in methanol. Sections were then blocked with appropriate blocking solution (NEN) and incubated with the respective primary antibodies in a humid chamber overnight at 4°C. After washing in Tris-buffered saline (TBS) containing 0.05% tween 20 (T-TBS), a biotinylated secondary antibody (Sigma) diluted in the blocking solution was applied for 1 h at room temperature. After washing in T-TBS, the sections were incubated with a streptavidin-peroxidase conjugate (NEN) for 30 min at room temperature. Signal amplification was performed by incubating in biotinyl tyramide working solution (NEN) and then sections were reincubated with a streptavidin-peroxidase conjugate. After washing with T-TBS, tissue bound peroxidase was detected using 0.2% 3,3'-diaminobenzidine in TBS. Sections were counterstained with hematoxylin. Primary antibodies (polyclonal) were goat anti-human recombinant TGase 1, and goat anti-guinea pig TGase 2 which were kindly donated by Dr. Chung SI (Aju University, Korea).

### Isolation and identification of crosslinked proteins

BAL fluid was boiled for 4 days at 110°C in solution containing 2% SDS, 10 M urea, 40 mM DTT. The boiled suspension was dialyzed against distilled water, centrifuged for 30 min at 15,000 rpm, 4°C. The pellet was then washed twice with distilled water and lyophilized. The insoluble precipitate resuspended in PBS was electrophoresed on 4-12% gradient SDS-polyacrylamide gel. Proteins were stained with Coomassie brilliant blue.

### Western blot analysis

After the protein concentration of BAL fluid was determined, equal amounts of the samples (30-50 μg) were separated on a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to a nitrocellulose membrane in an Xcell blot module (Novex, San Diego, CA). The membrane was incubated overnight with goat polyclonal anti-human rTGase 1, anti-guinea pig TGase 2 and anti-rabbit XIII, and monoclonal anti-isopeptide antisera. After washing the blots to remove unbound antibodies, the proteins of interest were visualized with the ECL system (Amersham, Buckinghamshire, UK).

### Statistics

There were 15 mice per group for experiments involving TBI and seperate additions of 6 mice 1 and 2 wk after 12 Gy TBI were used to examine thrombin-, TGase- and XIII-activities and crosslinked proteins in BAL fluid. Mean and standard error of the means were calculated for each group. Intergroup comparisons were made with Student's *t* test. *P* values of 0.05 or less were considered significant.

## Results

### Carbon monoxide uptake ($V_{CO}$)

The effect of graded single doses of TBI followed by BMT on $V_{CO}$ is shown in Figure 1. TBI and BMT caused a decrease in $V_{CO}$ that began during the first 1 month after treatment, and the $V_{CO}$ was significantly reduced below control levels.

### Thrombin activity

The thrombin activity in BAL fluid of control (unirradiated) mice was 7.08 U, whereas the activities of TBI-BMT mice were significantly higher than that of

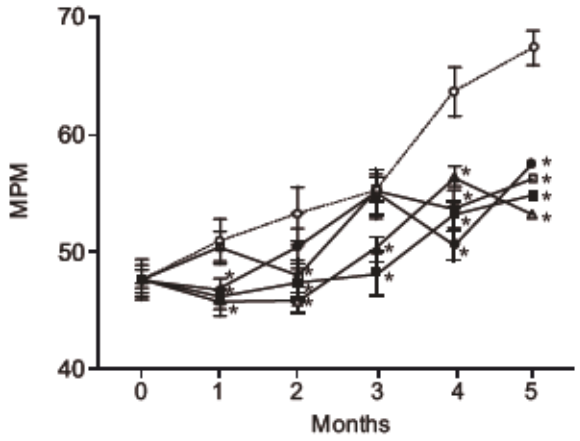


**Figure 1.** $V_{CO}$ (MPM=microliter per min) as a function of months after a range of single doses of TBI and BMT. —○—, control; —▲—, 12 Gy; —■—, 14 Gy; —□—, 16 Gy; —●—, 18 Gy. *$P < 0.05$.

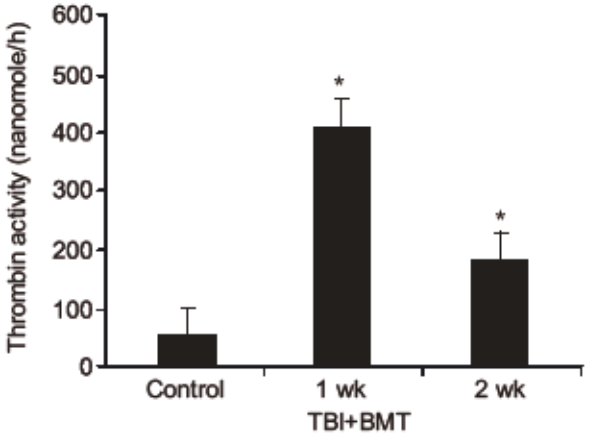


**Figure 2.** Thrombin activity in BAL fluid from irradiated mice. BAL was performed at 1 and 2 wks after irradiation. Thrombin activity was assayed fluorometrically with Boc-Val-Pro-Arg-MCA as a substrate, as described in Materials and Methods. Thrombin activity in BAL fluid was expressed as nanomoles of substrate per h. Values represent means±SEM, with $n = 3$. *$P < 0.05$.

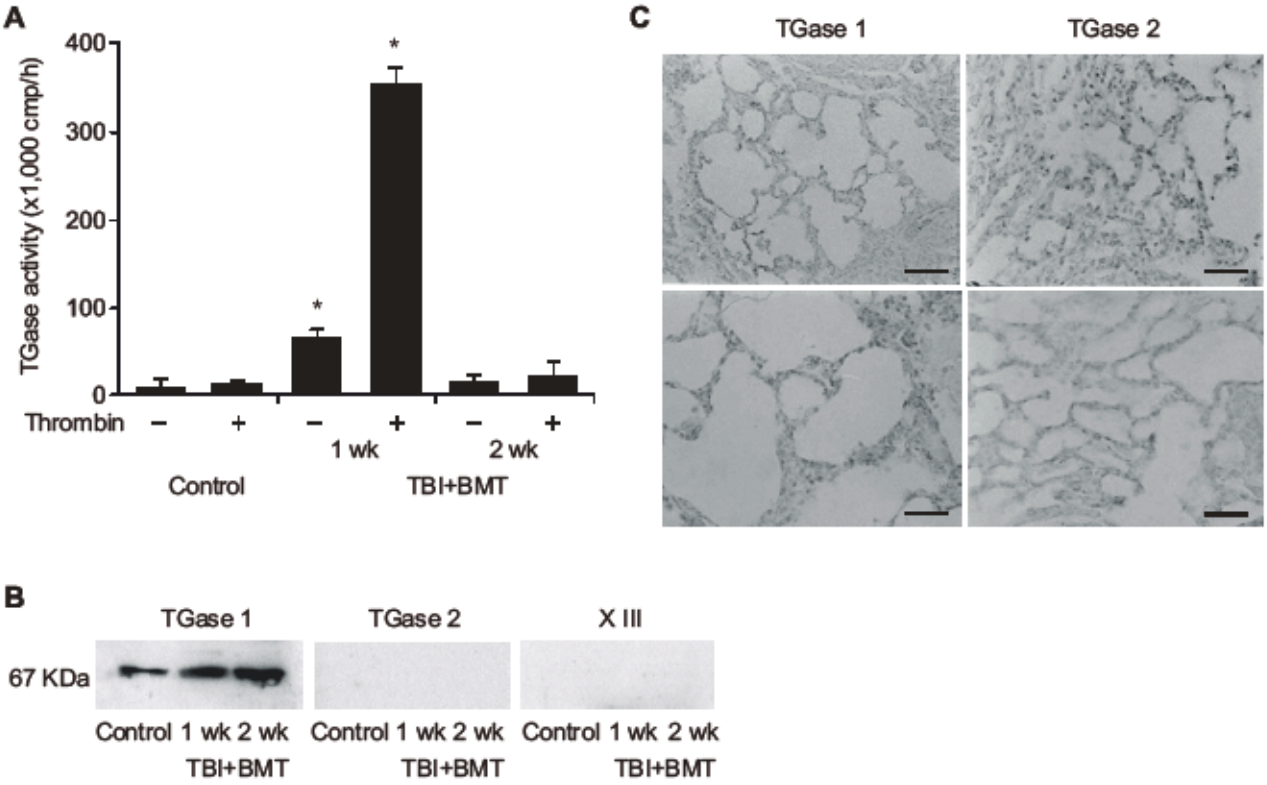


**Figure 3.** (A) TGase activity in BAL fluid from unirradiated and irradiated mice. BAL was performed at 1 and 2 wks after irradiation. TGase activity was measured by [$^{14}$C]-putrescine incorporated into dissuccinylated casein method, as described in Materials and Methods. *$P < 0.05$. (B) Expression patterns of TGases in BAL fluid from unirradiated and irradiated mice. Equal amounts (30 μg) of total proteins were analyzed by Western blot. (C) Immunohistochemistry of TGase 1 (a) and 2 (b). A portion of lung from unirradiated mice(upper panels) and mice 1 wk after TBI and BMT (lower panels), showing strong immunoreactivity, immunoperoxidase stain, scale bar, 100 μm.

control mice ($P < 0.05$), 55.2 U and 22.7 U at 1 wk and 2 wks after TBI of 12 Gy and BMT, respectively (Figure 2).

### TGase and XIII activities

As shown in Figure 3, TGase activities were detected only in BAL fluid from mice 1 wk after TBI of 12 Gy and BMT. The activities were never detected in the BAL fluid from unirradiated control mice and from mice 2 wk after TBI and BMT. The TGase activity was 63,000 cpm/h in the absence of thrombin, whereas the activity was 5.5 fold enhanced to 352,800 cpm/h in the presence of thrombin (A). Western blot analysis (B) showed expression of the 67 KDa TGase 1 protein in BAL fluid from control mice, and the mice 1 wk and 2 wks after TBI and BMT. TGase 2- and XIII-proteins were never expressed. Immunohistochemistry (C) revealed the immunoreactivities of both TGase 1 and TGase 2 in lung parenchymal cells from unirradiated (control) and irradiated mice. Thus, the increased TGase activity in the presence of thrombin from BAL fluid from the mice 1 wk after TBI and BMT appears to be TGase 1.

### Crosslinked protein

To investigate the nature of the insoluble protein in BAL fluid, it was extensively boiled in urea, and SDS-PAGE was performed. The insoluble protein did not migrate on 4-12% gradient SDS-PAGE, indicating that the protein is crosslinked (Figure 4A), and the isopeptide of the protein was confirmed by Western blot analysis (Figure 4B).

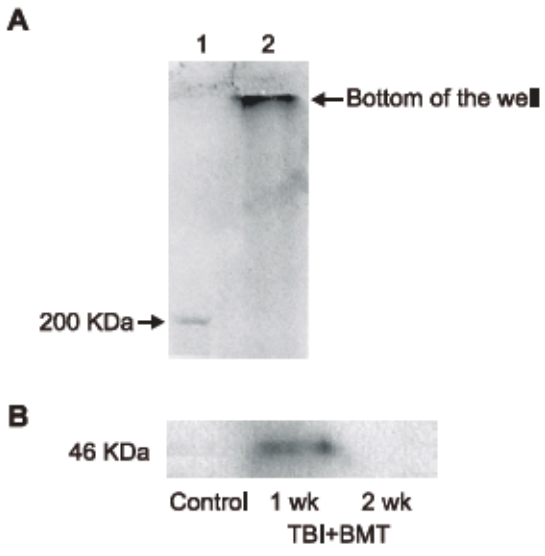


**Figure 4.** (A) SDS-PAGE of isopeptide linked protein. BAL fluid from mice 1 wk after TBI and BMT was boiled for 4 days in Tris buffer, pH 8.5 containing 10 M urea, 2% SDS, 40 mM DTT. Isolated isopeptide linked protein was electrophoresed on 4-12% gradient SDS-PAGE, and stained with Coomassie blue R. lane 1: molecular weight marker; lane 2: isopeptide linked protein. (B) Expression of isopeptide-bound crosslinked protein in BAL fluid from mice 1 wk after TBI and BMT by Western blot.

## Discussion

Although BMT is now a standard treatment for leukemias and other hematological malignancies, the preparatory TBI undertaken prior to BMT predisposes the patient to lung complications with a significant fatality rate. In the current study, we demonstrated that TBI-induced lung damage involves a decrease in $V_{CO}$, enhanced activities of thrombin and TGase 1 and the elevation of crosslinked protein in BAL fluid. $V_{CO}$ is known as a very sensitive indicator of detecting lung radiation injury (Travis *et al.*, 1979; DePledge *et al.*, 1981). Our results do show significant decrease of CO levels following irradiation and support the previous work of DePledge *et al.* (1981). Reduced $V_{CO}$ could be due to a decrease in the capillary-alveolar bed, uneven distribution of ventilation or perfusion, a change in the surface active properties of alveoli, or a change in the composition of alveolar septae (Roughton and Forster, 1957).

BAL (Steinberg *et al.*, 1993) is a melieu containing eluants of all the soluble components out of lung cells and allows for detecting any component changes that may be associated with the pathogenesis of radiation-induced lung damage. Based on such analysis, numerous lead candidates for lung radiation injury have been reported-TGF-β (Anscher *et al.*, 1995), IL-4 (Büttner *et al.*, 1997), IL-6 (Chen *et al.*, 2001), and thrombin (Huang *et al.*, 2001).

Thrombin activity greatly elevated in BAL fluid from thorax-irradiated rats has been reported to possess the proliferative activity of fibroblasts (Huang *et al.*, 2001). TGases are involved in the remodeling of granulation tissue through cross-linking (Haroon *et al.*, 1999). Most of the TGase1 in epithelial cells has been known to exist as a ≅100 kDa membrane-bound protein with low specific activity, but during terminal differentiation, some may be activated by proteolytic processing into a 67/33/10kDa form with high specific activity, and some intact low specific activity or higher specific activity processed forms are released into the cytosol (Steinert *et al.*, 1996). The ≅80 kDa TGase 2 enzyme is typically located in the cytosol and well-known to ubiquitously express tissue TGase (Folk and Finlayson, 1977). Cocuzzi and Chung (1986) reported the high expression of the TGase 1 in normal lung tissues. In the present study, thrombin- and TGase 1 activities and crosslinked proteins were identified in the BAL fluid of mice after TBI and BMT, and the

expression of TGase 1 and 2 could be seen in lungs from both unirradiated and irradiated mice, indicating that TGase 1 of BAL fluid from the mice 1 wk after TBI and BMT appears to be mobilized from the lung parenchymal cells.

In this study, the 67 KDa TGase 1 protein in BAL fluid from unirradiated mice and from mice 1 wk and 2 wks after TBI and BMT was confirmed by Western blot analysis. However, no enzyme activities were detected in the BAL fluid from unirradiated mice and from the mice 2 wks after TBI and BMT and remains to be clarified. It has been well recognized that the crosslinked protein formed by TGases in the early stage of inflammation confers mechanical stability which is known to be provisional matrix (Lorand and Conrad, 1984). Thus, although no TGase 1 activity in bronchoalveolar fluid from the mice 2 wks after TBI and BMT was observed, the crosslinked proteins formed by TGase 1 in the bronchoalveolar fluid from the mice 1 wk after TBI and BMT, appear to initiate fibrosis. In pulmonary damage, it is speculated that the activation of TGase 1 by thrombin, the $Ca^{2+}$-dependent enzyme responsible for catalyzing the crosslinks, together with enhanced levels of substrate proteins such as fibrin, fibrinogen, and fibronectin, could lead to the formation of stable excessive matrix crosslinked proteins.

This study provides evidence that the bronchoalveolar proteins crosslink by the thrombin-activatable TGase 1 in the early stages of radiation-induced lung damage followed by reduced lung function. This study seems to be the first to examine the expression of TGase 1 and the formation of $N^{\varepsilon}$-(γ-glutamyl) lysine crosslinks in BAL fluid of the injured lung after TBI and BMT.

**Acknowledgement**

This work was partly supported by Catholic Medical Center Grant 2003.

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