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Rhodostomin Inhibits the Transforming Growth Factor-β1-Enhanced Adhesion Activity of ROS 17/2.8 Osteosarcoma Cells

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Yang, R.-S. and Huang, T.-F. *Rhodostomin Inhibits the Transforming Growth Factor-β1-Enhanced Adhesion Activity of ROS 17/2.8 Osteosarcoma Cells.* Tohoku J. Exp. Med., 2000, **191** (3), 145-155 —— We have investigated the effect of transforming growth factor-β1 (TGF-β1) on the in vitro adhesion activity of the rat osteosarcoma cell lines (ROS 17/2.8) to extracellular matrix substrata, including fibronectin, type I and IV collagen, as well as laminin. The interaction of Arg-Gly-Asp (RGD) and rhodostomin, an RGD containing snake venom, with TGF-β1 on the cell adhesion was also evaluated. The results showed that incubation with various concentration of TGF-β1 (1-15 ng/ml) significantly increased the adhesion activity (1.4 to 2.5 folds) of ROS 17/2.8 to fibronectin and type I collagen ($p<0.01$), whereas the adhesion activity to laminin and type IV collagen was slightly elevated (1.1 to 1.5 folds). The peak effect of TGF-β1 on the cell adhesion occurred after pretreatment of ROS 17/2.8 with TGF-β1 for 6 hours. Treatment with Arg-Gly-Asp-Ser (RGDS) and rhodostomin effectively suppressed the TGF-β1-enhanced adhesion activity to fibronectin and type I collagen. This study demonstrated that the up-regulated cell adhesion activity of ROS 17/2.8 cells by the TGF-β1 can be inhibited by the rhodostomin. ———— osteosarcoma; cell adhesion; metastasis; TGF-β1; rhodostomin

Lung metastasis is the leading cause of the death in the patients of osteosarcoma which is a highly malignant tumor with a high metastatic potential. Tumor cell attachments to endothelium or to extracellular matrix (ECM) followed by penetration into surrounding tissue to form a second foci is a fundenmental step in the process of tumor implantation (MaCarthy et al. 1985; Albelda 1987; Aresu et al. 1991; Santala and Heino 1991; Lester and McCarthu 1992; MacDonald and Steeg 1993). Cell adhesion activities occur intercellularly as well as between cells and ECM substratum through various kinds of adhesion molecules and their receptors on the tumor cell surface (Albelda 1987; Dedhar et al.

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1987a, b; Freed et al. 1989; Fogerty et al. 1990; Nishimura 1990; Aresu et al. 1991; Lampugnani et al. 1991; Honn et al. 1992; Masi et al. 1992; MacDonald and Steeg 1993; Van Waes 1995). Previous studies demonstrated the presence of several kinds of integrin on the surface of different osteosarcoma cells (Dedhar et al. 1987a, b; Oldberg et al. 1988; Freed et al. 1989; Heino and Massague 1989; Chiang et al. 1995).

The cell adhesion is regulated by many kinds of cytokines, including transforming growth factor-β1 (TGF-β1) (Rhodes et al. 1955; Ignotz and Massague 1987; Dedhar 1989; Heino and Massague 1989; Santala and Heino 1991; Chakrabarty 1992; Huang and Chakrabarty 1994; Frank et al. 1996). Many tumors have been proved to produce TGF-β1, including sarcoma, squamous cell carcinoma, lung cancer, pancreatic cancer, breast cancer, hepatoma, renal cancer, adenocarcinoma (gastric and colon cancer), lymphoma and myeloma, etc.. TGF-β1 is closely related to many physiological and pathological processes, including the cell proliferation, adhesion, and mobilization, embryonic morphogenesis and differentiation, wound healing and remodeling, inflammation, etc. (Heine et al. 1987; Ignotz and Massague 1987; Roberts et al. 1988; Joyce et al. 1990a, b; Pelton et al. 1991; Bonewald et al. 1992; Mooradian et al. 1992; Andrew et al. 1993; Rhodes et al. 1995; Van Waes 1995; Frank et al. 1996). With regard to the tumor formation and progression, TGF-β1 has been proven to be involved in the formation of tumor matrix as well as angiogenesis (Heino and Massague 1989; Pfeilschifter et al. 1990; Daughaday and Deuel 1991; Bereta et al. 1992; Chakrabarty 1992; Mooradian et al. 1992; Kim and Ballock 1993; Huang and Chakrabarty 1994; Van Waes 1995). Furthermore, TGF-β1 is capable of regulating the expression of adhesion molecule and integrin receptors on the tumor cells, thus affecting the tumor cell adhesion and metastatic potential (Ignotz and Massague 1987; Santala and Heino 1991; Chakrabarty 1992; Huang and Chakrabarty 1994).

Up to the present, very few studies about the effects of TGF-β1 on the osteosarcoma cell adhesion have been done. Intravenous injection of the rat osteosarcoma (ROS) 17/2.8 cells has been shown to cause in vivo pulmonary metastasis in nude mice (Shirakawa et al. 1998). Since the metastasis cascade is highly related to the cell adhesion, the current study will investigate the effect of TGF-β1 on the adhesion properties of ROS 17/2.8, rat osteosarcoma cell lines. In addition, an increasing attention is being paid to the anti-adhesion, the antagonizing effects of synthetic peptide Arg-Gly-Asp-Ser (RGDS) and rhodostomin (an RGD containing peptide purified from the venom of the Malayan pit viper *Agkistrodon rhodostoma*) on the TGF-β1-treated cell adhesion activities will be evaluated.

Materials and Methods

Materials

ROS 17/2.8 cells were kindly supplied by Dr. L.T. Hou, Department of Dentistry, College of Medicine, National Taiwan University (originally from Dr. R.J. Majeska). Rhodostomin was purified from venom of *A. rhodostoma* as previously described (Huang et al. 1990). Synthetic peptide RGDS was obtained from Peninsula Laboratories (CA, USA), 2′, 7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein acetoxymethyl (BCECF-AM; Molecular Probes Inc., Eugene, OR, USA) has been used for fluorescence-based viability assessment in adherent cell cultures (Vaporciyan et al. 1993). TGF-β1, fibronectin (from bovine plasma), collagen type I (from calf skin) and type IV collagen, laminin (from basement membrane of mouse sarcoma), and collagenase (type I) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Methods

ROS 17/2.8 Cell culture. ROS 17/2.8 cells were cultured in 95% air-5% CO_2 using tissue culture-grade plastic flasks. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) medium which was supplemented with 20 mM N-2-hydroxyethlpiperazine-N′-2 ethane sulfonic acid (HEPES) and 10% heat-inactivated fetal calf serum, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (pH adjusted to 7.6). Based on trypan blue exclusion studies, the cell viability was greater than 96%.

Preparation of ECM coated Costar 96-well plates. The Costar 96-well plates were coated with various ECM, including fibronectin (30 μg/ml), laminin (15 μg/ml), Type I collagen (80 μg/ml), and Type IV collagen (80 μg/ml). The bovine serum albumin (BSA) was used for negative control. These coated wells were kept in laminar flow hood to air dry overnight, and washed with phosphate buffered-saline (PBS) immediately prior to adhesion assay.

Adhesion studies. The ROS 17/2.8 cells were used for experiment after reaching confluence. The ROS 17/2.8 cells (10^5 cells) were incubated with control buffer or various concentrations of serum-free TGF-β1 (0, 1, 3, 5, 10, 15 ng/ml) for different time periods (0-24 hours) to assess the effect of TGF-β1 on the ROS 17/2.8 cells adhesion to the aforementioned ECM substrata. Thereafter they were harvested and washed 3 times to remove residual TGF-β1. Finally they were resuspended in serum-free DMEM for cell adhesion studies. In this study, ROS 17/2.8 cells were incubated with BCECF-AM (fluorescent dye) (2 μg/ml) in Hanks balanced salt solution (HBSS) for 30 minutes at 37°C. Thereafter ROS 17/2.8 cells were washed in PBS and resuspended in HBSS. We used the ECM-coated Costar 96-well plates for adhesion studies. The BSA-coated wells in

the Costar 96-well plates was used for control during every experiment. Control or TGF-β1-treated ROS 17/2.8 cells were added to each well and incubated for 4 hours at 37°C. Then the nonadherent cells were removed by aspiration. The Costar 96-well plates were assessed using CytoFlour 2300 fluorescence plate reader (Millipore Co., Bedfold, MA, USA).

The dose and incubation time for the maximal response were determined. To investigate the effect of RGDS and rhodostomin on TGF-β1-treated cell adhesion activity, ROS 17/2.8 cells were treated with control buffer or TGF-β1 under the condition for its optimal effect, followed by washing and then addition of buffer, RGDS or rhodostomin and incubated for 30 minutes at 37°C before adhesion assay as mentioned previously. Each condition was run in quadruplicates and all experiments were repeated at least four times with comparable results. The results of cell adhesion were assessed as previously described.

Results

Effect of TGF-β1 on ROS 17/2.8 cells adhesion to ECM

The results showed that ROS 17/2.8 cells adhered well to the ECM-coated Costar 96-well culture plates. In addition, ROS 17/2.8 cells responded to TGF-β1 by a significantly increased adhesion activity to fibronectin and type I collagen in a dose-dependent pattern (1.4 to 2.5 folds, $p<0.01$) (Fig. 1). A concentration of TGF-β1 as low as 1 ng/ml was capable of increasing the cell adhesion potential, whereas the maximal effect occurred at a concentration of 3 ng/ml. On the other hand, the adhesion of ROS 17/2.8 cells to laminin and type IV collagen was

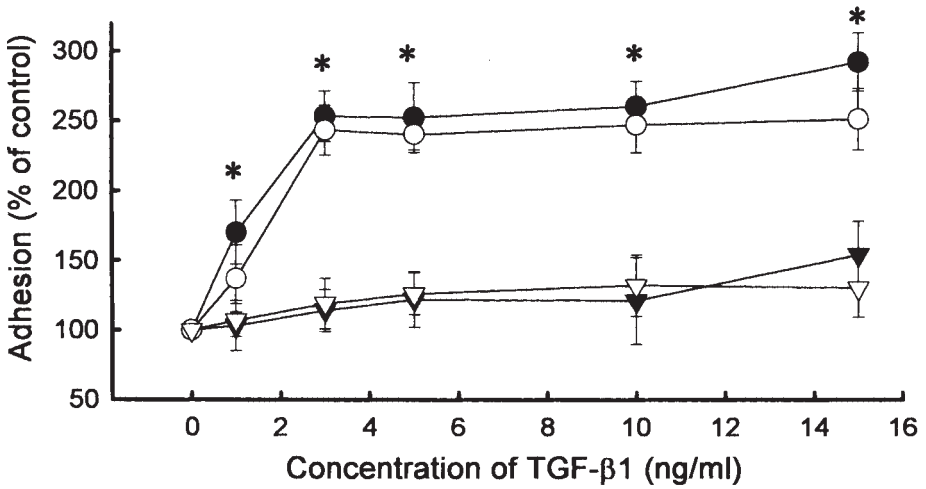

Fig. 1. Effect of TGF-β1 on the adhesion activities of ROS 17/2.8 osteosarcoma cells to fibronectin, type I collagen, type IV collagen and laminin. ROS 17/2.8 cells were pretreated with various concentration of TGF-β1 (1–15 ng/ml) for 6 hours at 37°C, and the cell adhesion was assessed after 4 hours. The results showed a potent stimulation effect on the ROS 17/2.8 cell adhesion to fibronectin and type I collagen in a dose-dependent pattern. Data are presented as mean$\pm$S.E.M. ($n=4$). *$p<0.01$, as compared to controls. ●, fibronectin; ○, type I collagen; ▲, type IV collagen; △, laminin.

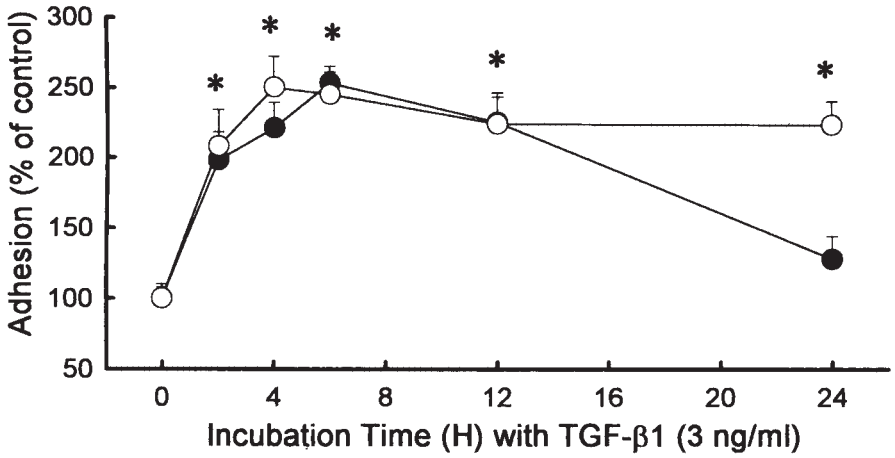

Fig. 2. Time course of treatment with TGF-β1 on the adhesion activities of ROS 17/2.8 cells to fibronectin and type I collagen. ROS 17/2.8 cells were pretreated with 3 ng/ml of TGF-β1 for various time periods (0–24 hours) at 37°C, and assessed for adhesion to fibronectin and type I collagen after 4 hours. The results showed a peak effect after 4 to 6 hour incubation of ROS 17/2.8 cells with TGF-β1. Data are presented as mean±S.E.M. ($n=4$). *$p<0.01$, as compared to controls. ●, fibronectin; ○, type I collagen.

slightly elevated (Fig. 1). Very few ROS 17/2.8 cells (<1% of control) were adherent to the wells coated with BSA. Thus the further investigation was performed on the fibronectin and type I collagen.

Incubation of ROS 17/2.8 cells with 3 ng/ml TGF-β1 at 37°C for different time periods revealed a bell-shaped pattern with the maximal response after 4 to 6 hours of incubation to fibronectin ($p<0.01$) (Fig 2). On the other hand, the significantly enhanced adhesion response to the type I collagen persisted up to 24 hours (Fig. 2).

Effect of RGDS and rhodostomin on TGF-β1-enhanced ROS 17/2.8 cell adhesion

TGF-β1, at a concentration of 3 ng/ml, caused the peak effect after incubating it with ROS 17/2.8 cells at 37°C for 6 hours. Thus we investigated the effect of RGDS and rhodostomin on the TGF-β1-stimulated ROS 17/2.8 cell adhesion under these optimal conditions. The results showed rhodostomin effectively suppressed the cell adhesion response to TGF-β1 at a concentration of 2.8 μg/ml (0.37 μM), either to fibronectin or type I collagen substrata ($p<0.01$). The synthetic RGDS had a similar antagonizing effect with the peak effect at 80 μg/ml (200 μM, $p<0.01$) (Fig. 3). On a molar basis, rhodostomin is about 540 times more potent than the synthetic RGDS in blocking the effect of TGF-β1-enhanced ROS 17/2.8 cell adhesion to ECM.

Discussion

This study demonstrated the potent stimulatory effect of TGF-β1 on the ROS 17/2.8 cells adhesion to the fibronectin and type I collagen substrata. However,

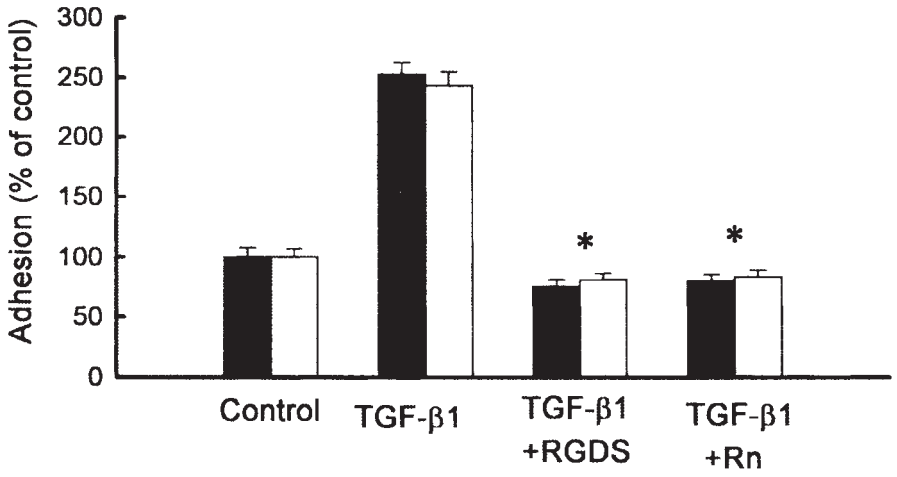

Fig. 3. Suppression of TGF-β1-stimulated ROS 17/2.8 cell adhesion by RGDS and rhodostomin. ROS 17/2.8 cells were treated with TGF-β1 (3 ng/ml, 6 hours, 37°C), then washed once and subsequently treated with buffer, rhodostomin (2.8 μg/ml) or RGDS (80 μg/ml) for 30 minutes at room temperature. Then the ROS 17/2.8 cells were assessed for their adhesion properties to type I collagen-coated and fibronectin-coated plates for 4 hours at 37°C. The results showed that rhodostomin potently blocked the TGF-β1-enhanced cell adhesion at a concentration of 2.8 μg/ml. The synthetic RGDS had a similar blockade effect with the peak effect at 80 μg/ml. On a molar basis, the antagonizing effect of rhodostomin is about 540 times more potent than RGDS. Data are presented as mean$\pm$S.E.M. ($n=4$). *$p<0.01$, as compared to thrombin-treated cells. ■, fibronectin; □, type I collagen.

the ROS 17/2.8 cell adhesion to laminin and type IV collagen was slightly elevated by the pretreatment with TGF-β1 on the ROS 17/2.8. A concentration of 3 ng/ml TGF-β1 caused the peak effect after incubating it with ROS 17/2.8 cells at 37°C for 6 hours. Such a finding supports the evidence of the high metastatic potential of the ROS 17/2.8 cells. In addition, the present study also demonstrated the potent antiadhesion effect of RGDS and rhodostomin. Therefore, TGF-β1 may play an important role on the regulation of cell adhesion and tumor metastatic potential of osteosarcoma.

Tumor cell adhesion through the interaction between the various adhesion molecules and their own integrins on the cell surface is the central event in the process of metastatic cascade (MaCarthy et al. 1985; Dedhar et al. 1987, 1989; Ignotz and Massague 1987; Oldberg et al. 1988; Freed et al. 1989; Heino and Massague 1989; Fogerty et al. 1990; Nishimura 1990; Aresu et al. 1991; Lampugnani et al. 1991; Santala and Heino 1991; Lester and McCarthu 1992; Masi et al. 1992). Various adhesion molecules and receptors have been demonstrated in these interactions, including laminin, type I and IV collagen, vitronectin, fibronectin, heparan sulfate proteoglycan, etc. (Aresu et al. 1991; Lester and McCarthu 1992; Masi et al. 1992). These interactions were regulated by various kinds of factors, including TGF-β1, a homodimer with the molecular weight (MW) of 25 kDa present in the platelet and bone matrix (Ignotz and

Massague 1987; Roberts et al. 1988; Dedhar 1989; Flanders et al. 1989; Heino and Massague 1989; Thompson et al. 1989; Goldring and Goldring 1990; Daughaday and Deuel 1991; Santala and Heino 1991; Chakrabarty 1992; Massague et al. 1992; Mooradian et al. 1992; Seitz et al. 1992; Huang and Chakrabarty 1994; Rhodes et al. 1995; Frank et al. 1996). In addition to its physiological functions, TGF-β1 has been shown to be closely related to the tumor progression. TGF-β1 in the tumor tissue may play an important role in the autocrine and paracrine regulation of the tumor cells. Many tumors was capable of producing TGF-β1, including sarcomas, squamous cell carcinoma, lung cancer, pancreatic cancer, breast cancer, hepatoma, renal cancer, adenocarcinoma (gastric and colon cancer), lymphoma and myeloma, etc. Furthermore, TGF-β1 has been shown to be reproduced by the osteosarcoma cells by the immunohistochemical study on the osteosarcoma tissues (Kolen et al. 1996; Yang et al. 1998). These local growth factors may have the paracrine effect to enhance the adhesion activities of the tumor cells during the metastatic cascade. Therefore the osteosarcoma may be a primary bone malignancy with a high potentential of pulmonary metastasis. These events may lead to earlier mortality in the clinical practice.

TGF-β1 is a potent stimulator of the matrix expression and integrin expression, depending on the types of the cells. With regard to the cell adhesion activities, previous studies demonstrated that TGF-β1 augments the tumor cell adhesion potential of the human pulmonary adenocarcinoma cells and human colon carcinoma cells (Chakrabarty 1992; Mooradian et al. 1992). Up-regulation of fibronectin and laminin receptors expression as well as increased fibronectin and laminin secretion by TGF-β1 has been demonstrated in human colon cancer cells (Huang and Chakrabarty 1994). Furthermore, TGF-β1 may increase the expression of ECM through elevated biosyhtnesis, decreased synthesis of protease, and stimulated synthesis of protease inhibitor, thus stimulate the adhesion potential (Heine et al. 1987; Roberts et al. 1988; Noda and Rodan 1989; Joyce et al. 1990a, b; Pelton et al. 1991; Chakrabarty 1992; Mooradian et al. 1992; Seitz et al. 1992; Kim and Ballock 1993; MacDonald and Steeg 1993; Newman 1993; Huang and Chakrabarty 1994; Walker et al. 1994). On the other hand, TGF-β1 has been shown to decrease the $\alpha 3\beta 1$ expression, increase $\alpha 5\beta 1$ and $\alpha 2\beta 1$ expression in MG-63 human osteosarcoma cells, resulting its selective loss of adhesion to laminin (Heino and Massague 1989; Santala and Heino 1991). In the current study, the TGF-β1 enhanced ROS 17/2.8 cell adhesion to fibronectin and type I collagen. Therefore, a higher blood levels of TGF-β1 or local concentration of TGF-β1 would increase the potential of the cell adhesion and resulting in metastasis. Furthermore, this TGF-β1-enhanced cell adhesion activities was effectively blocked by the RGDS and rhodostomin, suggesting the possible involvement of the integrins and RGD sequences. Further investigation of the expression of cell receptor or ECM synthesis is in progress.

The anti-adhesion effect on the tumor cell adhesion can be achieved through

the inhibition of the synthesis and expression of ECM or integrin, as well as blockade of the binding of adhesion molecules and their receptors. Rhodostomin, an RGD-containing snake venom peptide, can bind to its specific receptor associated with GP IIb/IIIa and effectively block the fibrinogen interaction (Heino and Massague 1989; Honn et al. 1992). Rhodostomin has been demonstrated to bind via its RGD sequence to many integrin receptors (i.e., $\alpha_{IIb}\beta_3$, $\alpha_v\beta_{23}$, $\alpha_5\beta_1$) expressed on the tumor cell surface and block the tumor cell adhesion to ECM (Chiang et al. 1995). In this study, we have demonstrated that both synthetic RGDS and rhodostomin could significantly suppress the TGF-β1-enhanced ROS 17/2.8 cell adhesion to fibronectin and type I collagen substrata. Rhodostomin was about 540 times more potent than RGDS in inhibiting TGF-β1-enhanced cell adhesion activity. Our current study suggest that rhodostomin may serve as a potent antimetastatic agent in blocking the TGF-β1-enhanced cell adhesion potential. Further investigation of antiadhesion therapy merits attention for the prevention of potential metastasis in the cancer patients.

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