Vol. 192, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1993 Pages 281-287

TUMOR NECROSIS FACTOR-α INCREASED THE INTEGRIN $\alpha 2 \beta 1$ EXPRESSION AND CELL ATTACHMENT TO TYPE I COLLAGEN IN HUMAN DERMAL FIBROBLASTS

Kyori Ezoe and Takashi Horikoshi

Department of Dermatology, Sapporo Medical College, S1, W16, Chuoku, Sapporo 060, JAPAN

Received February 22, 1993

SUMMARY : Cell adhesion molecules of human dermal fibroblasts play an important role in the processes of wound healing. The effects of tumor necrosis factor- α (TNF) on the expression of integrin $\beta 1$ subfamily in human dermal fibroblasts were examined. TNF preferentially induced the expression of $\alpha 2 \beta 1$ integrins, receptors for collagen and laminin, in a time and dose dependent manner. Cell attachment to type I collagen increased by the treatment with TNF. However, cell attachment to fibronectin and laminin was not increased. This TNF-induced cell attachment could be reduced significantly by anti-integrin $\alpha 2 \beta 1$ antibody. Antibodies against receptors other than $\alpha 2 \beta 1$ integrin did not significantly reduce cell attachment. These data suggest that the increased attachment of human dermal fibroblasts to type I collagen appears to be mediated predominantly through the augmentation of integrin $\alpha 2 \beta 1$ expression by TNF.

The wound healing comprises sequenced processes following tissue injury, which include the activation of platelets, monocyte / macrophages accumulation at the site of wound and the secretion of several kinds of cytokines . In response to cytokines, fibroblasts migrate, proliferate and synthesize extracellular matrix (ECM) proteins (1,2,3,4). Integrins of the $\beta 1$ subfamily bind to the ECM and are expressed on the surface of various kinds of cells (5,6,7). In particular, integrin $\alpha 2 \beta 1$ has been shown to be a main receptor for type I collagen and laminin (8,9,10). However, little is known about how the induction and function of integrin $\alpha 2 \beta 1$ is modulated by cytokines in the processes of wound healing. The concentration of TNF is known to increase in the tissue exudate of wounded sites (2). TNF has been shown to be a growth factor of human nontransformed fibroblasts (11,12). In this way, TNF is assumed to play a critical role in the regulation of cell growth and production of ECM by fibroblasts as elements of wound healing. Therefore, we investigated the effect of TNF on the expression of integrin $\beta 1$ subfamily on the surface of fibroblasts using fluorescence activated cell sorter (FACS) analyzer and the attachment of fibroblasts to collagen, fibronectin and laminin which have been likely to be one of the major elements in wound healing.

0006-291X/93 $4.00

MATERIALS AND METHODS

Cell cultures. Human dermal fibroblasts were isolated by the explant outgrowth from normal infant skin as previously described (13). Cells were maintained in Earle's Minimum Essential Medium (MEM: Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum (FCS: Flow Laboratories), penicillin (100U/ml) and streptomycin (100 μ g/ml) (GIBCO, Grand Island, NY) in humidified atmosphere containing 5% CO_2 at 37℃. Cells were detached from culture flasks by 0.05% trypsin / 0.53mM EDTA (GIBCO, Grand Island, NY) and subcultured at a 1: 3 split ratio. Five different strains were used for experiments between their 4th and 7th passage.

Monoclonal antibody. TS2/7 (mouse IgG1) antibodies (MoAbs): recognizing integrin $\alpha 1 \beta 1$ complex was purchased from T Cell Diagnostics. P1E6 (mouse IgG1): $\alpha 2$ subunit of integrin $\alpha 2 \beta 1$ complex and P1B5 (mouse IgG1): $\alpha 3$ subunit of integrin $\alpha 3 \beta 1$ complex from Oncogene Science. HP2/1 (mouse IgG1): $\alpha 4$ subunit of integrin $\alpha 4 \beta 1$ complex, SAM1 (mouse IgG2a): $\alpha 5$ subunit of integrin $\alpha 5 \beta 1$ complex and GoH3 (rat IgG2a): $\alpha 6$ subunit of integrin $\alpha 6 \beta 1$ complex from Immunotech S.A. DF5 (mouse IgG1): integrin $\beta 1$ subunit from LOCUS. Mouse IgG1 control, IgG2a control from Becton Dickinson, Fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse F(ab')2 IgG antibody and FITC-conjugated rabbit-anti-rat IgG antibody from DAKO.

Materials. Recombinant human TNF-α was supplied from Dainippon pharmaceutical Co., LTD (Osaka,Japan). The specific activity of TNF was 3×10^6 U/mg. Type I Collagen was obtained from Koken Co., LTD (Japan). Fibronectin and laminin were obtained from Collaborative Research Incorporated.

Flow cytometry. Fluorescence activated cell sorter (FACS) analysis was performed as previously described (14,15). Briefly, human dermal fibroblasts were seeded at $10^4/cm^2$ in $25cm^2$ flask. In order to minimize the effect of serum, cells were cultured in MEM containing low fetal calf serum (2%). 24h after seeding, attached cells were incubated with various concentrations of TNF for indicated periods. Then, cells were detached from the flask by treatment with 1mM EDTA to make single cell suspensions. After centrifugation at 180g for 10min at 4℃, cells were rinsed twice with phosphate buffered saline (PBS: Ca^{2+}, Mg^{2+} free) containing 0.1% sodium azide. Then cells were stained with 2 μ g of purified anti-integrin $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\beta 1$ MoAbs at 4℃ for 30min. As a negative control, isotype matched immunoglobulins were used. After rinsing three times with PBS, cells were then incubated with FITC-conjugated rabbit-anti-mouse F(ab')2 IgG and/or rat IgG antibody (1:20 diluted) for 30min at 4℃. Then cells were washed three times with PBS and fixed with 1% paraformaldehyde. Samples were analyzed using a FACS analyzer (Becton Dickinson) within 2 days after preparation. Data were expressed as follows.

$$\% \text{ increase of positive cells} = \frac{\% \text{ positive cells after treatment} - \% \text{ positive cells of control}}{\% \text{ positive cells of control}} \times 100\ (\%).$$

Cell attachment assay. Attachment assay was performed as previously described (16,17). Briefly, tissue culture plate (96well microplate) was coated by 10 μ g/ml of type I collagen, fibronectin and laminin for 24h at 4℃. Each well was rinsed three times with PBS and incubated with 1% bovine serum albumin for 2h in order to block non-specific cell binding. Before assay, cells were pretreated with 10U/ml of TNF for 48h in MEM containing 2%FCS. Cells were then detached from culture flasks by 1mM EDTA to make single cell suspensions in serum-free MEM at

the final concentration of 10^5cells/ml. The number of cells was counted by using Coulter Counter (Coulter Electronics, Inc.). 100 μl of cell suspensions were seeded onto collagen coated 96well microplates. 30 min after seeding, unattached cells were removed by rinsing three times with PBS. Then attached cells were stained with 0.5% crystal violet in methanol/water (1:4) for 15min. After washing three times with PBS, the dye incorporated into cells was eluted with 100 μl of 33% (v/v) acetic acid. The absorbance of eluted dye was read in 2-wave length microplate photometer (Corona, Japan) at 600nm. Cell number was directly proportional to the absorption of eluted dye ranging from $10^3 \sim 5 \times 10^4$ adherent cells (17).

For inhibition assay, cells stimulated or unstimulated for 48 hours by 10U/ml of TNF were incubated with 10 μ g/ml of anti-integrin $\alpha 1 \sim \alpha 6$ and $\beta 1$ MoAbs, mouse IgG1, IgG2a and PBS as control for 30 min. Then cells were allowed to attach to the collagen coated plates. The number of attached cells was calculated in the same way for cell adhesion assay. The absorbance of eluted dye at 600 nm of TNF-untreated control cells were set at 100%.

Statistical analysis. All experiments were repeated at least three times. Results were shown as mean ± standard deviations. Statistical significance of data was analyzed using the student t test.

RESULTS

Effect of TNF on the expression of integrin $\beta 1$ subfamily in human dermal fibroblasts.

The expression of integrin $\beta 1$ subfamily in untreated human dermal fibroblasts were assayed. Cells were cultured for 48 hr in the absence of TNF in MEM supplemented with 2% FCS. The basal expression of integrin $\beta 1$ subfamily ranged from 20 to 80% as shown in Fig. 1.

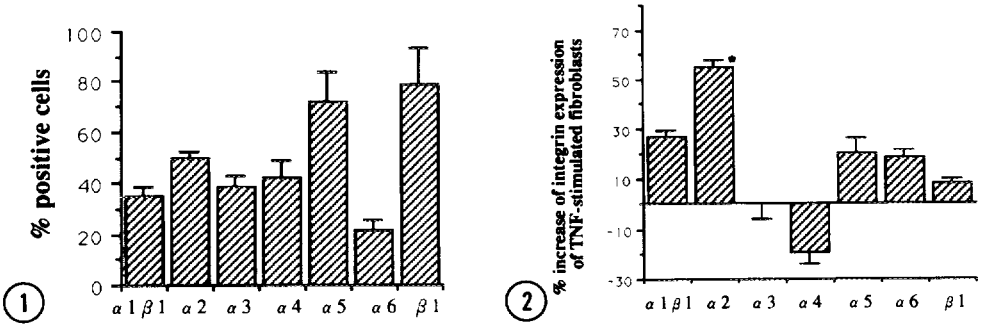

Fig.1. The basal expressions of integrin $\beta 1$ subfamily in non-treated human dermal fibroblasts. Percent positive cells with each integrin $\beta 1$ subfamily ranged from 20 to 80%. All experiments were repeated at least three times. Results were shown as mean ± standard deviations.

Fig.2. Effect of TNF on the expression of integrin $\beta 1$ subfamily in human dermal fibroblasts. When cells were treated with 10U/ml of TNF for 48h, the expression of $\alpha 2 \beta 1$ integrin, a receptor for collagen and laminin, was preferentially upregulated by 55% in comparison to that of control. Integrin $\alpha 4 \beta 1$, a receptor for fibronectin, was rather decreased below the control level. The other integrin $\beta 1$ subfamilies such as $\alpha 1 \beta 1$, $\alpha 5 \beta 1$ and $\alpha 6 \beta 1$ were also increased, but not significantly. All experiments were repeated at least three times. Results were shown as mean ± standard deviations. Statistical significance of data was analyzed using the student t test. * $p < 0.01$, compared to control.

The integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 4\beta 1$ were expressed in less than 50% of cells. Integrin $\alpha 5\beta 1$, a receptor for fibronectin, was expressed on the surface of more than 70% of cells. Integrin $\alpha 6\beta 1$, a receptor for laminin, was present in about 20% of cells. Integrin $\alpha 5\beta 1$, a receptor for fibronectin, was expressed most strongly in untreated control cells.

The expression of integrin $\beta 1$ subfamily was not augmented by treatment with TNF for the shorter periods of time than 24h (4, 8 and 12h, data not shown). When cells were treated with 10U/ml of TNF for 48 h, the expression of $\alpha 2\beta 1$ integrins, a receptor for collagen and laminin, were preferentially upregulated by 55 %. Integrin $\alpha 3\beta 1$, a receptor for fibronectin, laminin and collagen, was not altered. Integrin $\alpha 4\beta 1$, one of the receptors for fibronectin, was rather decreased below the control level. The other integrin $\beta 1$ subfamily such as $\alpha 1\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ were also increased. However, these upregulation were not significant as shown in Fig.2.

When cells were incubated with TNF at concentrations of 1, 100 and 1000U/ml for 48h, the increase of integrin $\alpha 2\beta 1$ expression was 43%, 57% and 50%, respectively (data not shown). These data indicated that the effects of TNF on the integrin $\alpha 2\beta 1$ expression were exerted in a time and dose dependent manner.

Effect of TNF on cell attachment to type I collagen, fibronectin and laminin.

As type I collagen, fibronectin and laminin are ligands for integrin $\beta 1$ subfamily (Table1), we investigated whether the expression of integrin stimulated by TNF might affect cell attachment to these extracellular matrix. When cells were treated with 10U/ml of TNF for 48h, the number of cells attached to type I collagen significantly increased by 87.4%. However, cell attachment to fibronectin and laminin were not increased (Fig. 3). These data showed that the augmentation of integrin $\alpha 2\beta 1$ by TNF was specifically related to the increased cell attachment to type I collagen, but not to laminin.

Effect of integrin $\alpha 2\beta 1$ MoAb on TNF-stimulated cell attachment to type I collagen.

TNF-untreated cell attachment to type I collagen was not reduced significantly by anti-integrin $\beta 1$ subfamily MoAbs (Table 2－I).

Table 1. Integrins of $\beta 1$ subfamily are heterodimers consisting of noncovalently α and β subunits. Most of the integrin $\beta 1$ subfamily bind to more than one ligand (5, 6, 7).

β Subunit	α Subunits	Ligands
	$\alpha 1$	LM, CL
	$\alpha 2$	CL, LM
$\beta 1$	$\alpha 3$	FN, LM, CL
	$\alpha 4$	FN, VCAM-1
	$\alpha 5$	FN,
	$\alpha 6$	LM

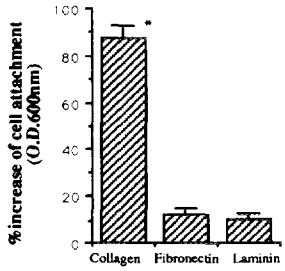

Fig.3. Effect of TNF on cell attachment to type I collagen, fibronectin and laminin. When cells were treated with 10U/ml of TNF for 48h, the number of cell attachment to type I collagen significantly increased by 87.4%, compared to that of TNF-untreated control. Cell attachment to fibronectin and laminin was not increased. Results were shown as mean ± standard deviations. Statistical significance of data was analyzed using the student t test.
* $p < 0.01$, compared to TNF-untreated control.

When cells pretreated with TNF for 48h were incubated for 30 min with anti-integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$ and $\beta 1$ MoAbs, cell attachment to type I collagen was significantly inhibited only by anti-integrin $\alpha 2\beta 1$ MoAb. Antibodies against the other $\beta 1$ integrin subfamily including $\alpha 1\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$ and $\beta 1$ did not significantly inhibit the attachment of TNF-treated cells to type I collagen (Table 2－II).

Table 2. Effect of anti-integrin $\beta 1$ subfamily MoAbs on cell attachment to type I collagen. Cells pretreated with or without 10U/ml of TNF for 48h were incubated with anti-integrin $\alpha 1$～$\alpha 6$ and $\beta 1$ MoAbs and with either isotyped matched IgG or PBS as control for 30min. (I) The attachment of non-treated cells was not reduced significantly by anti-integrin $\beta 1$ subfamily MoAbs. (II) The percent attachment of TNF-treated cells was significantly inhibited by 34.7% only in the presence of $\alpha 2\beta 1$ MoAb. The other anti-integrin $\beta 1$ family MoAbs did not reduced TNF-stimulated cell attachment. The number of cell attachment to collagen was determined by 2-wave length microplate photometer at 600nm. All experiments were repeated at least three times. Results were shown as mean ± standard deviations. Statistical significance of data was analyzed using the student t test. * $p < 0.01$ compared to TNF-treated cells.

	TNF	
	(I) Untreated (%inhibition)	(II) Treated (%inhibition)
Control	100 ± 9.4	187.4 ± 19.7
$\alpha 1$	95.7 ± 12.9 (4.3)	154.5 ± 23.8 (17.7)
$\alpha 2$	80.2 ± 10.1 (19.8)	122.7 ± 12.3* (34.7)
$\alpha 3$	91.6 ± 11.4 (8.4)	170.7 ± 14.5 (8.10)
$\alpha 4$	86.2 ± 12.5 (13.8)	179.8 ± 24.0 (4.2)
$\alpha 5$	94.2 ± 5.9 (5.8)	171.6 ± 21.1 (8.9)
$\alpha 6$	86.4 ± 12.3 (13.6)	169.2 ± 17.3 (9.8)
$\beta 1$	87.9 ± 16.0 (12.1)	156.2 ± 13.4 (16.8)

DISCUSSION

TNF stimulates the growth of normal human fibroblasts (11,12) and is related to the fibrosis or lung injury induced by bleomycin (18). TNF induces the expression of non-integrin cell adhesion molecule, such as intercellular adhesion molecule-1 (ICAM-1) in fibroblasts (19) and increases the adhesion of human synovial fibroblasts to T lymphocyte via the upregulation of ICAM-1 (20). In this way, TNF plays a critical role in the expression of cell adhesion molecules and regulate the interactions of cell to cell and cell to ECM recognition. However, the effects of TNF on the expression of integrin β 1 subfamily has not been elucidated yet.

In our results, TNF significantly increased the expression of integrin $\alpha 2 \beta 1$ in a time and dose dependent manner. Furthermore, TNF increased the number of cells attached to type I collagen. However, TNF did not increased the adhesion to fibronectin and laminin. TNF-stimulated attachment to collagen decreased significantly only in the presence of anti-integrin $\alpha 2 \beta 1$ MoAb. Although the cell adhesion molecules in addition to integrin $\alpha 2 \beta 1$ might be involved in the process of the cell attachment to type I collagen, our findings suggest that TNF-induced attachment to type I collagen is mediated predominantly through the expression of integrin $\alpha 2 \beta 1$.

At the injured tissue, resident fibroblasts might be exposed to various kinds of cytokines released by the coagulated platelets and the activated white blood cells (21). It is possible to assume that the upregulation of integrin $\alpha 2 \beta 1$ on fibroblasts by TNF is one of the crucial processes that promote their migration and adhesion to the exposed matrix, especially to collagen.

TNF has been shown to induce the production of prostaglandins by human fibroblasts (22,23,24). In our preliminary results, the effect of TNF on the induction of integrin $\alpha 2 \beta 1$ and cell attachment to type I collagen were inhibited by the indomethacin, cyclooxygenase inhibitor. Thus, wound healing is carried out through very complicated processes because a number of factors are involved, such as cytokines, extracellular matrix, cell adhesion molecules and intracellular metabolites. Further study would be needed to elucidate the mechanism of wound healing.

Acknowledgments: Makoto Takahashi, M.D., Professor, Department of Dermatology, Sapporo Medical College, and D.M.Carter, M.D. & Ph.D., Professor & Senior physician, Laboratory for Investigative Dermatology, The Rockefeller University, gave encouragement and assisted in a critical reading of this manuscript. This work was supported in part by grant No. 04670644 (T.H.) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Cotran, R. S., Kumar, V., Robbins, S. L. (1989) Robbins Pathologic Basis of Disease, 4th ed. Philadelphia, WB Saunders, 71-86.
2. Ford, H. R., Hoffman, R. A., Wing, E. J., Magee, D. M., Simmons, R. L., (1989) Arch. Surg.124, 1422-1428.
3. Rappolee, D. A., Mark, D., Banda, M. J., Werb, Zena. (1988) Science 241, 708-712.

4. Stoker, M., Gherard, E. (1991) Biochim. Biophys. Acta 1072, 81-102.
5. Springer, T. A. (1990) Nature 346, 425-433.
6. Hemler, M. E. (1990) Annu. Rev. Immunol. 8, 365-400.
7. Shimizu, Y., Van Seventer, G. A., Horgan, K. J., Shaw, S. (1990) Nature 345, 250-253.
8. Elices, M. J., Hemler, M. E. (1989) Proc. Natl. Acad. Sci. USA 86, 9906-9910.
9. Staatz, W. D., Walsh, J. J., Pexton, T., Santoro, S. A. (1990) J. Biol.Chem. 265, 4778-4781.
10. Staatz, W. D., Fok, K. F., Zutter, M. M., Adams, S. P., Rodriguez, B.A., Santoro, S. A. (1991) J. Biol. Chem. 266, 7363-7367.
11. Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M.A. Jr., Shepard, H. M. (1985) Science 230, 943-945.
12. Vilcek, J., Palombella, V. J., Destefano, D. H., Swenson, C., Feinman, R., Hirai, M., Tsujimoto, M. (1986) J. Exp. Med. 163, 632-643.
13. Paye, M., Nusgens, B. V., Lapiere, C.M. (1987) Eur. J. Cell. Biol. 45, 44-50.
14. Norris, D. A., Lyons, M. B., Middleton, M. H., Yohn, J. J., Kashihara-Sawami, M. (1990) J. Invest. Dermatol. 95, 132-138.
15. Marlor, C. W., Webb, D. L., Bombara, M. P., Greve, J. M., Blue, M.L. (1992) Am. J. Pathol. 140, 1055-1060.
16. Luca, D. M., Tamura, R. N., Kajiji, S., Bondanza, S., Rossino, P., Cancedda, R., Marchisio, P.C., Quaranta, V. (1990) Proc. Natl. Acad. Sci. USA 87, 6888-6892.
17. Majewski, S., Hunzelmann, N., Schirren, C. G., Mauch, C., Aumailley, M., Krieg, T. (1992) J. Invest. Dermtol. 98, 86-91.
18. Piguet, P. F., Collart, M. A., Grau, G.E., Kapanci, Y., Vassalli, P. (1989) J. Exp. Med. 170, 655-663.
19. Rothlein, R., Czajkowski, M., O'neill, M. M., Marlin, S. D., Mainolfi, E., Merluzzi, V. J. (1988) J. Immunol. 141, 1665-1669.
20. Krzesicki, R. F., Fleming, W. E., Winterrowd, G. E., Hatfield, C. A., Sanders, M. E., Chin, J. E.(1991) Arthritis. Rhum. 34, 1245-1253.
21. Ginsberg, M.H., Loftus, J.C., Plow, E.F. (1988) Thromb. Haematostasis. 59, 1-6.
22. Dayer, J. M., Beutler, B., Cerami, A. (1985) J. Exp. Med. 162, 2163-2168.
23. Elias, J. A., Gustilo, K., Baeder, W., Freundlich, B. (1987) J. Immunol. 138, 3812-3816.
24. Hori, T., Yamanaka, Y., Hayakawa, M., Shibamoto, S., Tsujimoto,M., Naoto, Oku., Ito F.(1991) Biochem. Biophys. Res. Commun. 174, 758-766.