BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **219,** 261–265 (1996)
ARTICLE NO. 0215

VLA-5-Mediated Interaction with Fibronectin Induces Cytokine Production by Human Chondrocytes

Ikuho Yonezawa,*,[1] Kazunori Kato,† Hideo Yagita,† Yasuo Yamauchi,* and Ko Okumura†

**Departments of Orthopedic Surgery, and †Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received December 28, 1995

Adhesion molecules of the integrin family, including very late activation antigens (VLA), have been implicated in various cellular functions. In this study, we investigated the contribution of integrin-mediated interaction with ECM proteins to the cytokine gene expression in human chondrocytes. Human articular chondrocytes expressed VLA-1, -2, -3 and -5 on the cell surface, and could adhere to various ECM proteins, especially to fibronectin (FN). Furthermore, the production of GM-CSF and IL-6 was potently induced by culturing chondrocytes on immobilized FN. This stimulative effect of FN was completely inhibited by an anti-integrin α5 chain mAb, as well as by anti-integrin β1 chain mAbs. These results indicate an important role of the VLA-5-mediated interaction with FN in regulating inflammatory cytokine production by human articular chondrocytes. © 1996 Academic Press, Inc.

β1 integrins, alternatively called very late activation antigens (VLA), are members of the integrin superfamily and each consists of a common β1 subunit and non-covalently associated one of at least nine different α subunits (1,2). These molecules are widely distributed in a variety of cells and contribute to the binding to multiple ligands including fibronectin (FN), collagen (CL), laminin (LM) and vascular cell adhesion molecule-1 (VCAM-1). β1 integrin-mediated cell-cell and cell-extracellular matrix (ECM) interactions have been implicated in many vital functions in development, hemostasis and immune responses (1–4). Several studies with VLA α or β chain-specific antibodies demonstrated that ECM binding via VLA molecules provided a co-stimulatory activation signal to lymphocytes, platelets and fibroblasts (5–7). In a previous study, we demonstrated that FN receptor integrins expressed on rat mast cells play an important role in regulating mast cell activation both *in vitro* and *in vivo* (8). It has been shown that human and bovine articular chondrocytes express ECM receptor integrins and adhere to collagen and fibronectin (9,10). However, little has been known about the physiological functions of β1 integrins on human chondrocytes. In the present study, we examined the involvement of β1 integrin-mediated interaction with a variety of ECM proteins in regulating the inflammatory cytokine production by human articular chondrocytes.

MATERIALS AND METHODS

Preparation of chondrocytes. Human chondrocytes were isolated from articular cartilage dissected at the time of joint replacement surgery for femoral neck fractures by digestion with pronase and collagenase as described by Zafarullah et al. (11). Briefly, an articular cartilage was sliced into small pieces and digested by incubation for 2h with 0.4% actinase E (Koken, Tokyo) in DMEM, followed by digestion with 0.025% collagenase P (Boehringer Mannheim, FRG) overnight at 37°C. Isolated chondrocytes were washed and resuspended in serum-free AIM-V medium (Gibco, Grand Island, NY).

Antibodies. The following mouse and rat mAbs against human integrin α and β subunits were used: TS2/7 (anti-α1), P1E6 (anti-α2), SE-β3 (anti-β3) and GoH3 (anti-α6) were purchased from T Cell Diagnostics Inc., Chemicon, Sumitomo Electoric Industry and Immunotech, respectively. SG73 (anti-α4), KH/33 (anti-α5), SG19 (anti-β1), 4B4 (anti-β1) and J143 (anti-α3) were kindly donated by Dr. K. Miyake (Saga Medical School), Dr. C. Morimoto (Institute of Medical Science, University of Tokyo) and Dr. S. Furusawa (Hiroshima University). FITC-conjugated anti-mouse IgG antibody was purchased from Caltag (San Francisco, CA).

[1] To whom correspondence should be addressed. Fax: +81-(0)3-3813-0421.

0006-291X/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Immunofluorescence. To examine the expression of integrins on human chondrocytes, isolated chondrocyte was preincubated in DMEM supplimented with 10% FCS overnight at 37°C. The cells were harvested and incubated with 1 μg of the indicated mAb for 30 min at 4°C, followed by FITC-conjugated anti-mouse IgG. After washing twice with PBS, the cells were analyzed on FACScan (Becton Dickinson, San Jose, CA).

Adhesion assay. Human FN (Gibco), human type I collagen (CL-I) (Chemicon, Temecula, CA), human type II collagen (CL-II) (Chemicon), human vitronectin (VN) (Chemicon) and human laminin (LM) (Mallinckrodt Speciality Chemical Inc., Chesterfield, MO) were distributed at a concentration of 10 μg/ml in PBS onto 24-well plate overnight at 4°C, followed by blocking with 1% BSA in PBS for 2h at 37°C. Isolated chondrocytes were loaded with 10 μM 2′, 7′-bis(2-carboxyethyl) carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM) (Wako, Tokyo) in AIM-V for 1h and washed twice with the medium. BCECF-labeled cells were transferred to 24-well plate coated with various ECM proteins and incubated at 37°C for 2h. Non-adherent cells were removed by gentle washing with the medium. Then the adherent cells were solubilized with 0.5% TritonX-100 in PBS and fluorescent intensity was measured on a Fluoroskan II (Flow Laboratories, Inc, CA). For the blocking study, labeled chondrocytes were preincubated with the indicated mAbs for 30 min before transferring to ECM-coated plates.

RT-PCR and ELISA for cytokines. Expression of various cytokine genes was detected by the RT-PCR detection method as described previously (12). In brief, isolated chondrocytes (1×10^6 cells/ml in AIM-V medium) were cultured on 6-well plate coated with ECM proteins for 48h at 37°C. After the incubation, cytoplasmic RNA was extracted using Trizolve (Biotex Labo Inc., Houston, TX). First-strand cDNA was synthesized by using oligo(dT) primer and SuperScript reverse transcriptase (Gibco) from 5 μg of each RNA sample. PCR primers specific for human IL-1β, IL-6, GM-CSF, TNFα, and GAPDH were purchased from Clonotech (Palo Alto, CA). For PCR, cDNA was amplified in a reaction mixture containing 2 μM each of the 5′ and 3′ primers, 225 μM dNTP, 1U of AmpliTaq DNA polymerase (Perkin Elmer Cetus Corp., Emeryville, CA). PCR was performed in a DNA thermal cycler (Perkin Elmer) for 30 cycles (94°C for 1min, 60°C for 2 min, 72°C for 3min) followed by a 10 min extension at 72°C. The PCR products were subjected to electrophoresis on 2% agarose gels, and visualized under UV. Cell-free supernatant was also collected after the culture of chondrocytes on ECM proteins and tested for the concentration of human IL-6 and GM-CSF by ELISA using a kit Quantikine (R&D system, MN) according to the manufacturer's instruction.

RESULTS

Expression of integrin molecules on human chondrocytes. To characterize the expression of ECM receptor integrins on human chondrocytes, we first examined the reactivity of mAbs against various integrins α and β subunits with freshly isolated human articular chondrocytes by flow cytometry. As shown in Fig. 1, chondrocytes expressed a high level of α5 and β1, which constitute VLA-5, as well as low levels of α1, α2 and α3. In contrast to the high expression of β1 subunit, the mAb against β3 subunit reacted with chondrocytes very weakly, indicating that expression of β3 integrins including VNR and GPIIb/IIIa is marginal.

Adhesion of human chondrocytes to ECM proteins. We next investigated the adherence of human

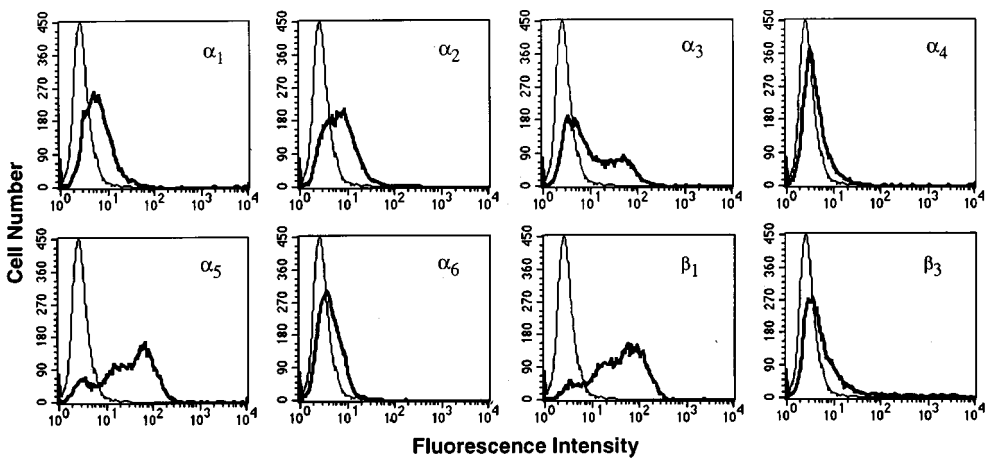

FIG. 1. Expression of integrin α and β subunits on isolated human articular chondrocytes. Cells were stained with mAbs specific for the indicated human integrin α or β subunits, followed by FITC-conjugated anti-mouse IgG. Immunofluorescence was analyzed on FACScan (bold lines). Thin lines indicate background staining with the second antibody alone.

chondrocytes to various ECM proteins, including FN, CL, VN and LM. As shown in Fig. 2, chondrocytes efficiently adhered to FN or LM and, to lesser extents, to CL or VN as compared to BSA. Similar results were also obtained with chondrocytes from some other patients (data not shown).

Cytokine gene expression and secretion by chondrocytes cultured on FN. We next examined the effect of cell adherence to ECM proteins on the expression of inflammatory cytokine genes in chondrocytes. Isolated chondrocytes were cultured on ECM-coated plates for 48 h. Expression of GM-CSF mRNA was specifically induced on FN but not on BSA, CL, LM, VN (Fig. 3A). IL-6 mRNA was induced by FN, LM, and VN but not by CL. In contrast, no apparent induction of IL-1β or TNFα mRNA was observed on any ECM tested (data not shown).

To examine whether FN-stimulated chondrocytes can secrete GM-CSF and IL-6, concentration of these cytokines in the supernatant was determined by ELISA. As shown in Fig. 3B, GM-CSF and IL-6 were produced by chondrocytes when cultured on FN but not on BSA. These results indicate that the interaction with FN induces some cytokine production by human chondrocytes.

Inhibitory effect of anti-VLA-5 mAbs on FN-mediated cell adherence and cytokine production. To identify the predominant FN receptor on human chondrocytes that leads to the cytokine production, we examined the contribution of FN-binding integrins, including VLA-3, VLA-4 and VLA-5, by estimating the inhibitory effect of mAbs against these integrin subunits. As shown in Fig. 4A, adhesion of chondrocytes to FN was completely inhibited by an anti-α5 mAb as well as by two anti-β1 mAbs, whereas anti-α3, α4 or β3 mAb did not affect. Moreover, the FN-induced expression of IL-6 mRNA was blocked by the addition of anti-α5 or β1 mAb but not by anti-α3 mAb (Fig. 4B). Similar results were also obtained in the secretion of IL-6 as estimated by ELISA (data not shown). These results clearly indicate that VLA-5 ($\alpha5\beta1$) on human chondrocytes acts as the predominant FN receptor that regulates IL-6 and GM-CSF production.

DISCUSSION

In the present study, we revealed a crucial role of the VLA-5-mediated interaction with FN in regulating the cytokine production by human articular chondrocytes. Several recent studies have indicated that articular chondrocytes express integrins and adhere to ECM proteins such as FN and CL (9,10). Our present observation that chondrocytes expressed a high level of VLA-5 and adhered to FN, was consistent with the previous result that VLA-5 was the most prominent in bovine chondrocytes (13). However, the physiological functions of VLA-5 expressed on human chondro-

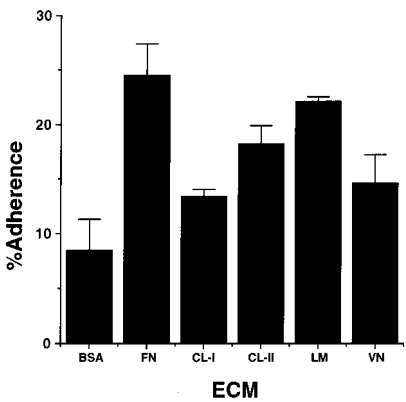

FIG. 2. Adhesion of human chondrocytes to ECM proteins. Fluorescence-labeled human chondrocytes were added to the wells coated with the indicated ECM proteins. After 2h incubation, non-adherent cells were removed by gentle washing and the fluorescent level of adherent cells was measured by fluorometer.

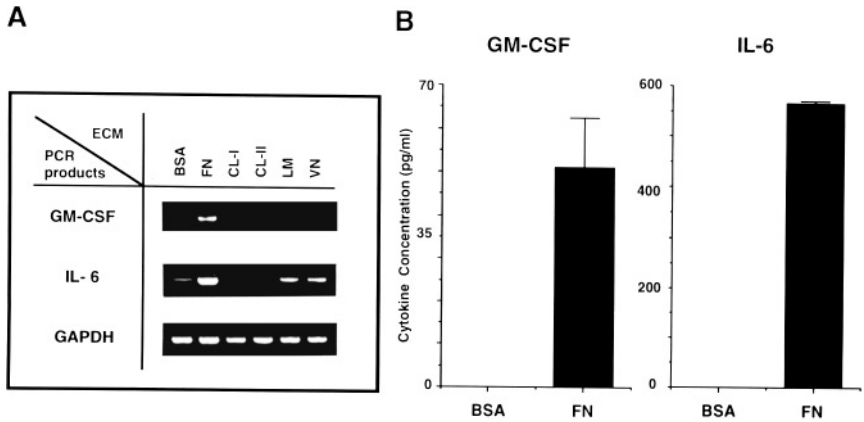

FIG. 3. Cytokine gene expression and production by chondrocytes cultured on FN. (A) Cytoplasmic RNA prepared from human chondrocytes, which had been cultured in serum-free medium on BSA-, FN-, CL-I-, CL-II-, LM- or VN-coated plate for 48h, was subjected to RT-PCR detection of GM-CSF, IL-6 and GAPDH mRNA. (B) After the culture of human chondrocytes on FN or BSA, cell-free supernatants were collected and tested for the concentration of human IL-6 and GM-CSF by ELISA.

cytes has not been elucidated yet. Therefore, we here indicated that ligation of VLA-5 with FN induces production of inflammatory cytokines, such as IL-6 and GM-CSF, by chondrocytes.

Several evidence for VLA-5 as a signal transducing receptor have been shown in a variety of cell types. Shimizu et al. demonstrated the costimulation of proliferative response of $CD4^{+}$T cells by the interaction of VLA-5 and VLA-4 with FN (14). FN binding to VLA-5 has been also shown to induce the AP-1 transcriptional factor necessary for IL-2 production (15). Furthermore, Werb et al. investigated the effect of ligation of VLA-5 on metalloproteinase gene expression in synovial fibroblasts (16). We also reported that degranulation of rat mast cell was enhanced by the interaction of VLA-5 with immobilized FN. Consistent with these results, cytokine production was induced in chondrocytes when cultured on FN-coated plate, indicating that VLA-5 acts as a signaling receptor on articular chondrocytes. However, the intracellular events that leads to cyto-

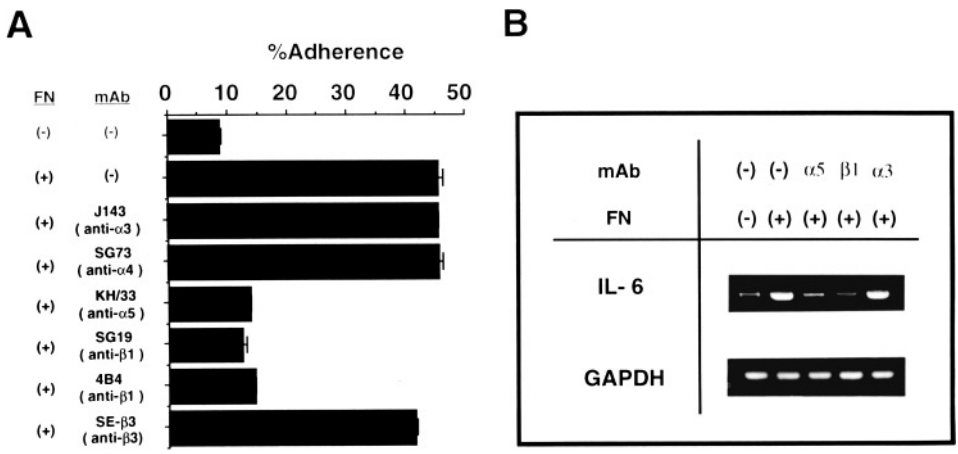

FIG. 4. Inhibitory effect of anti-$\alpha5/\beta1$ mAbs on FN-mediated cell adherence and cytokine production. (A) Inhibition of adhesion to FN by chondrocytes. The indicated mAbs were added to the adhesion assay at a final concentration of 10 μg/ml. Data represent mean + SD of triplicate wells. (B) Inhibition of cytokine gene expression. Chondrocytes were cultured on FN-coated plate with or without mAb against the indicated integrin α or β subunit. After culturing for 48 h, cytoplasmic RNA was prepared and subjected to RT-PCR detection of IL-6 mRNA.

kine production, such as tyrosine phosphorylation and activation of nuclear factors, remains to be determined by further studies.

Previous reports have shown that the production of IL-6 and GM-CSF by human chondrocytes was induced by exogeneous TNFα or IL-1 (17,18). We also observed the induction of IL-6 and GM-CSF production by IL-1β was further up-regulated when chondrocytes were cultured on FN-coated plate (data not shown). It has been suggested that secretion of these cytokines by chondrocytes may accerelate the release of matrix degrading enzymes and thus was implicated in the pathogenesis of osteoarthritis (OA) and rheumatoid arthritis (RA) (19,20). Moreover, it has been shown that VLA-5 expression was increased in osteoarthritic cartilage as compared with normal cartilage and that concentration of FN was increased in synovial fluid with deposition of FN on the surface of the articular cartilage in OA (10,21). VLA-5 appears to play a key role in regulating cytokine production by chondrocytes. Therefore, the interference of the VLA-5-mediated interaction with FN by using some chemical drugs or antagonistic molecules, such as antibodies and peptides, might be useful for quenching the overproduction of harmful cytokines in human articular cartilage.

ACKNOWLEDGMENTS

We thank Drs. Yoriaki Kaneko and Shinichiro Kobayashi for technical assistances. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, the Ministry of Health (Japan), and Japan Orthopaedics and Traumatology Foundation, Inc. (No. 0072).

REFERENCES

1. Hemler, M. E. (1992) *Annu. Rev. Immunol.* **8,** 365–400.
2. Palmer, E. L., Rüegg, C., Ferrando, R., Pytela, R., and Sheppard, D. (1993) *J. Cell Biol.* **123,** 1289–1297.
3. Hynes, R. O. (1992) *Cell* **69,** 11–25.
4. Williams, D. A., Rios, M., Stephens, C., and Patel, V. P. (1991) *Nature* **352,** 438–.
5. Shimizu, Y., van Seventer, G. A., Horgan, K. J., and Shaw, S. (1990) *Immunol. Rev.* **114,** 109–143.
6. Matsuyama, T., Yamada, A., Kay, J., Yamada, K. M., Akiyama, S. K., Schlossman, S. F., and Morimoto, C. (1989) *J. Exp. Med.* **170,** 1133–1148.
7. Yamada, A., Nojima, Y., Sugita, K., Dang, N. H., Schlossman, S. F., and Morimoto, C. (1991) *Eur. J. Immunol.* **21,** 319–325.
8. Yasuda, M., Hasunuma, Y., Adachi, H., Sekine, C., Sakanishi, T., Hashimoto, H., Ra, C., Yagita, H., and Okumura, K. (1995) *Int. Immunol.* **7,** 251–258.
9. Dürr, J., Goodman, S., Potocnik, A., von der Mark, H., and von der Mark, K. (1993) *Exp. Cell Res.* **207,** 235–244.
10. Loeser, R. F., Carlson, C. S., and McGee, M. P. (1995) *Exp. Cell Res.* **217,** 248–257.
11. Zafarullah, M., Martel-Pelletier, J., Cloutier, J.-M., Gedamu, L., and Pelletier, J.-P. (1992) *FEBS letters* **306,** 169–172.
12. Miyake, S., Yagita, H., Maruyama, T., Hashimoto, H., Miyasaka, N., and Okumura, K. (1993) *J. Exp. Med.* **177,** 863–868.
13. Loeser, R. F., Carlson, C. S., and McGee, M. P. (1995) *Exp. Cell Res.* **217,** 248–257.
14. Shimizu, Y., van Seventer, G. A., Horgan, K. J., and Shaw, S. (1990) *J. Immunol.* **145,** 59–67.
15. Yamada, A., Nikaido, T., Nojima, Y., Schlossman, S. F., and Morimoto, C. (1991) *J. Immunol.* **146,** 53–56.
16. Werb, Z., Tremble, P. M., Behrendtsen, O., Crowley, E., and Damsky, C. H. (1989) *J. Cell Biol.* **109,** 877–889.
17. Malfait, A. M., Verbruggen, G., Veys, E. M., Lambert, J., De Ridder, L., and Cornelissen, M. (1994) *J. Rheumatol.* **21,** 314–320.
18. Alsalameh, S., Firestein, G. S., Oez, S., Kurrle, R., Kalden, J. R., and Burmester, G. R. (1994) *J. Rheumatol.* **21,** 993–1002.
19. Shinmei, M., Masuda, K., Kikuchi, T., and Shimomura, Y. (1989) *J. Rheumatol.* **16,** 32–34.
20. Nietfeld, J. J., Wilbrink, B., Helle, M., van Roy, J. L. A. M., den Otter, W., Swaak, A. J. G., and Huber-Bruning, O. (1990) *Arthritis Rheum.* **33,** 1695–1701.
21. Jones, K. L., Brown, M., Ali, S. Y., and Brown, R. A. (1987) *Ann. Rheum. Dis.* **46,** 809–815.