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INTERACTION OF FIBRONECTIN (FN) CELL BINDING FRAGMENTS AND INTERLEUKIN-8 (IL-8) IN REGULATING NEUTROPHIL CHEMOTAXIS

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SUMMARY. This study investigated the possible interaction of FN fragments in regulating IL-8-mediated neutrophil chemotaxis in vitro using Neuroprobe microchambers. Human neutrophil suspensions were incubated with purified FN fragments or an RGD-containing peptide and allowed to migrate in response to chemotactically active concentrations of human recombinant IL-8. The 120-kD fragment of FN containing the RGD sequence or an RGD peptide (GRGDSP) inhibited IL-8-mediated neutrophil chemotaxis; however, these RGD peptides did not inhibit neutrophil chemotaxis in response to other chemotactic agents. Furthermore, FN fragments not containing the RGD sequence had no effect on IL-8-mediated chemotaxis. These data suggest that directed migration of neutrophils in response to IL-8 is inhibited in the presence of cell-binding fragments of FN and may represent a local mechanism for terminating neutrophil migration at areas of tissue injury.

In response to the localized production of chemoattractants, peripheral blood neutrophils migrate across the endothelium into tissues and come into contact with components of the extracellular matrix. Fibronectin (FN) is a glycoprotein found both in the plasma and the extracellular matrix possessing a variety of biological effects on leukocytes, including stimulation of adherence, phagocytosis, cell migration and regulation of respiratory burst (1-6). Our laboratory has previously shown that fragments of FN containing the cell-binding domain are released from stimulated endothelial cells in culture and these FN fragments can promote neutrophil migration (7).

Interleukin-8 (IL-8) is a member of the alpha-chemokine family of low molecular weight peptides produced by mononuclear phagocytes, endothelial cells and other

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cell types in response to inflammatory agonists (8,9). IL-8 has been shown to exhibit multiple effects on neutrophils including stimulation of chemotaxis (10), adherence (11), degranulation (10) and rapid metabolic responses (12,13). Since endothelial cells release into the media both chemotactic chemokines (14) and fragments of extracellular matrix proteins (7) in response to inflammatory stimuli, we investigated the possible interactions of purified fragments of FN and recombinant IL-8 on neutrophil chemotactic function.

MATERIALS AND METHODS

REAGENTS. The 72 amino acid form of recombinant IL-8 (rIL-8) was purchased from Peprotech, Rocky Hill, MD. Intact human fibronectin dimer (440 kD) and fibronectin fragments (120 kD, 70 kD and 29 kD) were kindly provided by Dr. John Kaplan of the Albany Medical College and were isolated from human plasma as previously described (15,16). Zymosan, N-formyl-methionyl-leucyl-phenylalanine (FMLP) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co, St. Louis, MO. Zymosan activated serum (ZAS) was prepared as previously described (17) and used as a positive control in chemotactic experiments. Microchemotaxis chambers were purchased from Neuroprobe, Cabin John, MD. Dulbecco's modified Eagles medium (DMEM) and an RGD containing peptide (GRGDSP) were purchased from Gibco, Grand Island, NY.

PREPARATION OF HUMAN NEUTROPHIL SUSPENSIONS. Human peripheral blood neutrophils were isolated from heparinized blood drawn from volunteer normal donors using an approved human protocol as previously described (17). Isolated neutrophils were determined to be >95% viable by trypan blue exclusion and >98% pure by differential staining.

NEUTROPHIL CHEMOTAXIS ASSAY. Neutrophil chemotaxis was assayed in a 48 well Neuroprobe microchemotaxis system using 3 μm PVP-free polycarbonate filters. For each variable tested, neutrophils suspended in DMEM were added to triplicate wells at a concentration of 3.2×10^4 cells/50 μl (4000 cells/mm^2 of filter area) to the top wells and allowed to incubate for 45 minutes at 37°C. Human rIL-8 was added to the bottom wells to stimulate neutrophil chemotaxis. Fibronectin (FN) fragments or the GRGDSP peptide were added either to the neutrophil suspensions prior to adding cells to the top wells or were added to the lower wells containing rIL-8. Following incubation, counts were made of the number of neutrophils that migrated to the bottom of the filters in a 100 mm^3 grid using light microscopy with 400 x magnification. A chemotactic index (CI) was calculated as the average number of cells migrating relative to a positive (10% ZAS in 1% BSA in DMEM) and negative (1% BSA in DMEM) controls by the formula:

$$CI = \frac{(\text{experimental value - negative control})}{(\text{positive control - negative control})} \times 100.$$

RESULTS

Figure 1 illustrates the effect of either human rIL-8 or a 120 kD cell-binding fragment of FN to stimulate neutrophil chemotactic activity. Figure 1a illustrates

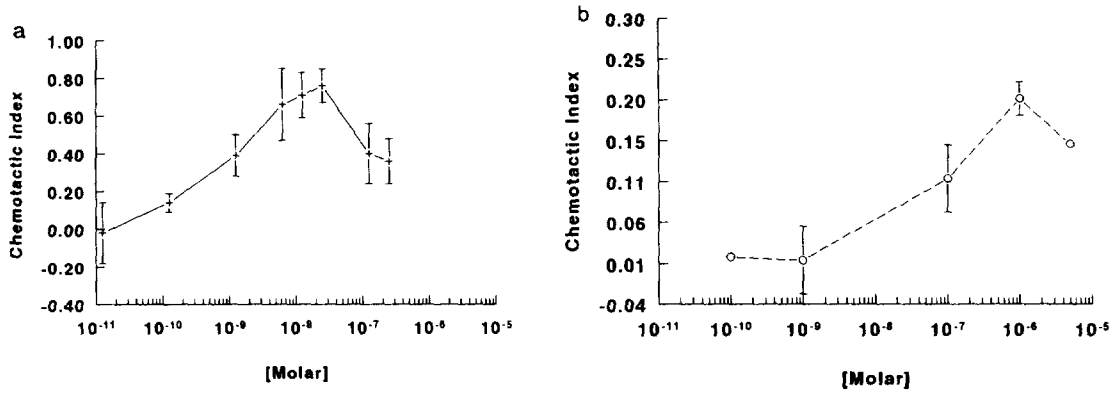

Figure 1. Human neutrophils (3.2×10^4 cells/50 μl) were added to the upper wells of triplicate microchemotaxis chambers in the presence of increasing concentrations of either a) recombinant IL-8 (rIL-8) or b) 120 kD cell binding FN fragment for 45 min at 37°C. Results are expressed as chemotactic index relative to a positive (10% zymosan activated serum in media) and negative (1% BSA in media) controls. Data represent the mean ± SEM of 6-9 experiments.

that rIL-8 increased neutrophil chemotaxis in a dose-dependent manner between 10^{-10} and 10^{-8} M. Concentrations of rIL-8 $>10^{-7}$ M produced a decrease in the chemotactic index. Figure 1b shows that the 120 kD cell-binding fragment of FN also stimulated neutrophil migration at concentrations ranging between 10^{-7} and 5 x 10^{-6} M. As can be seen, neutrophil migration in response to the cell-binding FN fragment was not as potent as to rIL-8.

Figure 2 illustrates the specificity of the cell-binding fragment of FN to inhibit rIL-8 mediated neutrophil chemotaxis. The addition of the 120 kD FN (10^{-6} M) to the neutrophil suspension reduced IL-8 mediated chemotaxis by approximately 50%. However, the addition of a 70 kD fragment of FN that did not contain an RGD sequence resulted in no inhibition of IL-8 mediated chemotaxis. In addition, the gelatin-binding domain fragment of FN (29 kD) also had no effect on rIL-8 mediated neutrophil chemotaxis (data not shown).

We have previously shown that pretreatment of neutrophils with an RGD-containing peptide inhibited 120 kD FN fragment mediated chemotaxis (7). In the present study, we examined the ability of an RGD peptide (GRGDSP) to inhibit neutrophil chemotaxis in response to optimum chemotactic concentrations of zymosan-activated serum (ZAS), FMLP, rIL-8 and the 120 kD or 70 kD fragments of FN (Figure 3). A 10 min preincubation of neutrophils with 1 mM GRGDSP did not affect either ZAS or FMLP mediated neutrophil chemotaxis but significantly reduced neutrophil migration in response to rIL-8 and the 120 kD FN fragment.

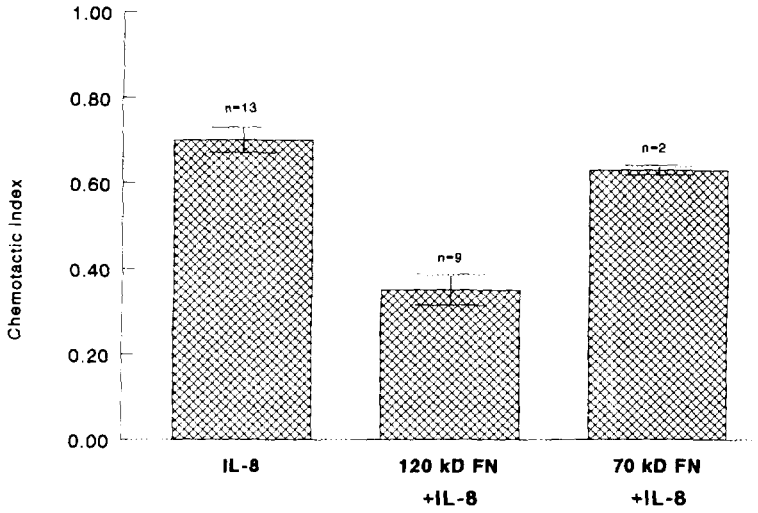

Figure 2. Human neutrophils were incubated in the absence or presence of either 120-kD FN (10^{-6} M) or 70-kD (10^{-6} M) FN fragments in the upper wells prior to measuring IL-8-mediated neutrophil chemotaxis.

Neutrophils can be desensitized to the migratory effects of various chemoattractants by prior exposure to the chemoattractant. We compared the desensitization effects of rIL-8 and the 120 kD FN fragment on rIL-8 mediated chemotaxis. As shown in Figure 4, a 10 min preincubation of neutrophils with rIL-8 significantly reduced the chemotactic response to IL-8 in the lower wells (i.e., homolo-

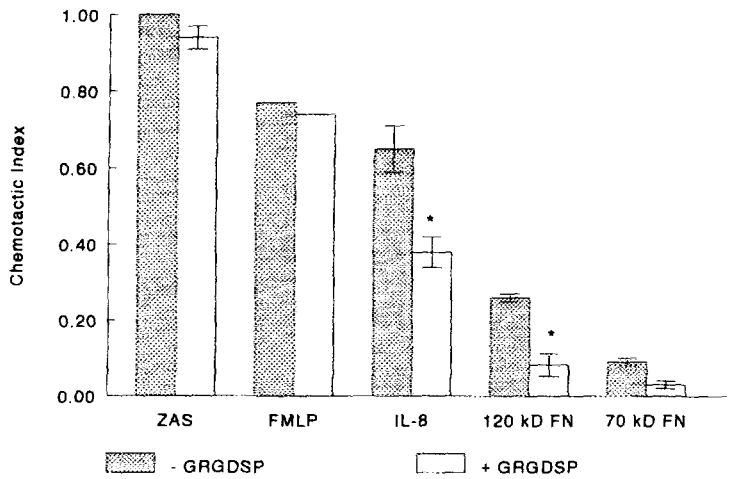

Figure 3. Neutrophils were preincubated with 1 mM GRGDSP for 10 min prior to measuring chemotaxis in response to 10% zymosan-activated serum (ZAS), FMLP (10^{-8} M) 120 kD FN (10^{-6} M) and rIL-8 (10^{-8} M). Data represents the mean ± SEM of triplicate samples from a representative experiment (n=3). *P<.05.

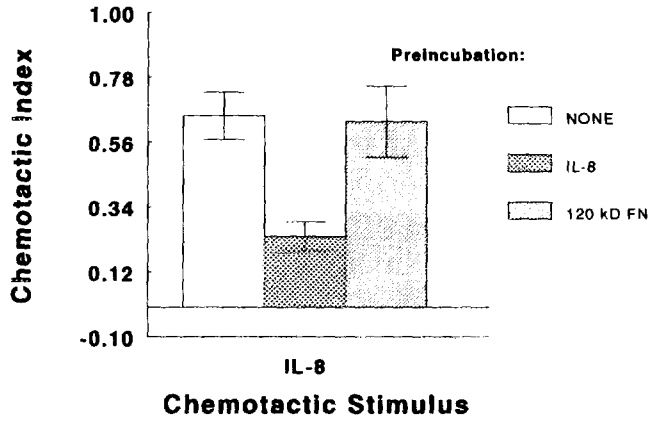

Figure 4. Neutrophils were preincubated with media (control), IL-8 (10^{-8} M) or 120 kD FN (10^{-6} M) for 10 min, washed to remove soluble chemoattractant, and added to the upper wells. rIL-8 was then added to the lower wells and neutrophil chemotaxis measured. Data represent the mean ± SEM of triplicate samples from a representative experiment (n=4).

gous desensitization). However, preincubation of neutrophils with the 120 kD FN fragment did not alter rIL-8 mediated chemotaxis. When a similar experiment was performed to desensitize the migratory response to the 120 kD FN fragment, preincubation of neutrophils with rIL-8 did not reduce migration to the 120 kD FN fragment (data not shown).

DISCUSSION

Interleukin-8 (IL-8) is a member of the chemokine family of low molecular proteins that stimulate neutrophil chemotaxis and activation. The present study demonstrates that a cell-binding fragment of FN can inhibit neutrophil chemotaxis in response to IL-8. The inhibitory effect of the 120 kD FN fragment was specific for the region of the FN molecule that included the RGD-containing cell-binding region since two other fragments of FN, a 29 kD gelatin-binding and a 70 kD amino terminal fragment, had no effect on inhibiting neutrophil chemotaxis. Previous studies in our laboratory (7) have demonstrated that the cell-binding fragment of FN could stimulate neutrophil migratory activity but that the intact FN molecule or FN fragments not containing the RGD sequence had no direct effect on neutrophil motility. In the present study, preincubation of neutrophils with GRDGSP selectively inhibited both IL-8 and 120 kd FN-mediated neutrophil migration while having no effect on inhibiting neutrophil migration in response to either FMLP or zymosan activating serum. These results demonstrate that

peptides containing the RGD sequence do not non-selectively inhibit neutrophil chemotaxis in response to all chemoattractants. The mechanism for this inhibition by RGD-containing peptides remains to be identified.

Many acute inflammatory processes are characterized by an early influx of peripheral blood neutrophils that subsequently declines and is followed by a later phase of monocyte emigration. Mechanisms that govern the transition between these two phases of leukocyte migration are poorly understood. It is well known that neutrophil migration can be inhibited or desensitized to a given chemoattractant by prior exposure to the agonist (i.e., homologous desensitization) or by prior exposure to selected structurally unrelated chemotactic factors (i.e., heterologous desensitization). In the present study, IL-8 mediated chemotaxis was inhibited by prior exposure of neutrophils to rIL-8 but not when neutrophils were preincubated with the 120 kD FN fragment. The absence of heterologous desensitization suggests that the 120 kD FN fragment is not interfering directly with IL-8 ligand/receptor interactions. Furthermore, in experiments not shown, incubation of neutrophils with rIL-8 did not interfere with the stimulation of neutrophil migration in response to the 120 kD FN fragment suggesting no cross desensitization by rIL-8.

Although several studies (18,19,20) have shown that the directed migration of leukocytes is stimulated by proteolytic fragments of extracellular matrix proteins, recent studies have demonstrated that the multicomponent subendothelial basement membrane can inhibit or delay neutrophil activation. For example, Matzner et al. (21) have demonstrated that neutrophils can adhere to an endothelial cell-derived basement membrane with no detectable release of lysosomal enzymes, respiratory burst activity or chemotactic activity in response to soluble stimulators. Furthermore, studies from our laboratory (1) and others (22,23) have shown that proteins isolated from the subendothelial basement membrane affect neutrophil function selectively. For example, purified fibronectin, but not collagen type IV or laminin, inhibited agonist induced respiratory burst activity of neutrophil suspensions.

In conclusion, the present findings provide evidence that IL-8 mediated neutrophil chemotaxis can be inhibited by cell-binding fragments of fibronectin. These results support a possible role for extracellular matrix protein fragments that contain the RGD cell-binding sequence in terminating the migration of chemokine-stimulated neutrophils at areas of tissue injury.

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REFERENCES

1. Gudewicz, P.W., Beezhold, D.H., Van Alten, P. and Molnar, J. (1982) J. Reticuloendothel. Soc. 32, 143-154.

2. Wall, R.T., Copper, S.L. and Kosek, J.C. (1982) Exp. Cell. Res. 140, 105-109.

3. Hoffstein, S.T., Gennaro, D.E. and Manzi, R.M. (1985) Inflammation 9, 425-437.

4. Monboisse, J.C., Garnotel, R., Randoux, A., Duter, J. and Borel, J.P. (1991) J. Leukocyte Biol. 50, 373-380.

5. Kownatski, E. and Uhrich, S. (1991) Agents and Actions 32, 41-44.

6. Nathan, C.F. (1987) J. Clin. Invest. 80, 1550-1560.

7. Odekon, L.E., Frewin, M.B., Del Vecchio, P., Saba, T.M. and Gudewicz, P.W. (1991) Immunology 74, 114-120.

8. Herbert, C.A., Lucinskas, F.W., Kiely, J.M., Luis, E.A., Darbonne, W.C., Bennet, G.L., Liu, C.C., Obin, M.S., Gimbrone, Jr., M.A. and Baker, J.B. (1990) J. Immunol. 145, 3033-3040.

9. Baggiolini, M., Walz, A. and Kunkel, S.L. (1989) J. Clin. Invest. 84, 1045-1049.

10. Paveri, P., Walz, A., Dewald, B. and Baggiolini, M. (1988) J. Exp. Med. 167, 1547-1559.

11. Carveth, H.J., Bohnsack, J.F., McIntyre, T.M., Baggiolini, M., Prescott, S.M., and Zimmerman, G.A. (1989) Biochem. Biophys. Res. Comm. 162, 387-393.

12. Roberts, P.J., Pizzey, A.R., Khwaja, A., Carver, J.E. Mire-Sluis, A.R. and Linch, D.C. (1993) Brit. J. of Hematology 84, 586-594.

13. Daniels, R.H., Finnen, M.J., Hill, M.E. and Lackie, J.M. (1992) Immunology 75, 157-163.

14. Strieter, R.M., Kunkel, S.L., Showell, H.J., Remick, D.G., Phan, S.H., Ward, P.A. and Marks, R.M. (1989) Science 243, 1467-1469.

15. Pirschbacher, M.D., Hayman, E.G., and Rouslahti, E. (1981) Cell, 259-267.

16. Blystone, S.D. and Kaplan, J.E. (1992) J. Biol. Chem. 267, 3968-3975.

17. Gudewicz, P.W., Weaver, M.B., Del Vecchio, P.J. and Saba, T.M. (1989) J. Lab. Clin. Med. 113, 708-716.

18. Bryant, G., Rao, C.N., Bretani, M., Martins, W., Lopes, J.D., Martin, S.E., Liotta, L.A. and Schiffmann, E. (1987) J. Leuk. Biol. 41, 220-227.

19. Laskin, D.L., Kimura, T., Sakakibara, S., Riley, D.J. and Berg, R.A. (1986) J. Leuk. Biol. 39, 255-266.

20. Norris, D.A., Clark, R.A.F., Swigart, L.M., Huff, J.C., Weston, W.L. and Howell, S.E. (1982) J. Immunol. 129, 1612-1618.

21. Matzner, Y., Vlodavsky, I., Michaeli, R.I. and Eldor, A. (1990) Exp. Cell Res. 189, 233-240.

22. Fehr, J., Moser, R., Leppert, D. and Groscurth, P. (1985) J. Clin. Invest. 76, 535-542.

23. Hoffstein, S.T., Gennaro, D.E. and Manzi, R.B. (1985) Lab. Invest. 52, 515-522.