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Expression of membrane-associated C-reactive protein by human monocytes: indications for a selectin-like activity participating in adhesion

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We have shown previously that rat liver macrophages (Kupffer cells) express a membrane-bound form of C-reactive protein (mCRP) on their surface which is identical to a galactose-specific particle receptor activity. We now establish the presence of mCRP on human monocyte-macrophages using immunocytochemistry with an anti-neoCRP specific monocloncal antibody and RNA-RNA *in situ* hybridization to demonstrate the presence of CRP-specific mRNA. Concomitant with mCRP expression, cells exhibit galactose-dependent uptake of partieles coated with lactosylated bovine serum albumin. Adhesion experiments on fibronectin-coated surfaces that mCRP on human blood monocytes may act as a selectin-like adhesion molecule, mediating initial carbohydrate-specific contacts which are followed by peptide-specific recognition via integrin receptors.

Keywords: membrane-associated C-reactive protein, human monocytes, adhesion molecule

Introduction

The acute phase protein C-reactive protein (CRP) has been shown to play an important role in protection against bacterial infections, and CRP or CRP-derived peptides were shown to modify various activities of immune effector cells [1]. In the presence of Ca^{2+} CRP is known to bind a number of ligands, i.e. phosphorylcholine [2, 3], the complement component Clq [4], fibronectin [5, 6], chromatin, histones [7, 8], and carbohydrates [9, 10] among others. Recently we were able to demonstrate that a galactose-specific receptor activity on rat liver macrophages (Kupffer cells) is identical to a membrane-associated form of CRP (mCRP) [11]. The molecular structure of mCRP appears to differ from the circulating form of CRP, as it is not recognized by antibodies against native serum CRP but by antibodies binding a denatured form of serum CRP [12] termed 'neoCRP' representing CRP subunits [13]. These findings are in accord with the demonstration of the presence of CRP-specific membrane receptor in macrophage plasma membranes [14, 15]. CRP has been shown to bind to immobilized plasma fibronectin pFn [6] and examination of the binding and uptake of fibronectin-coated particles by isolated rat liver macrophages had demonstrated that the lectin-like binding site of CRP recognizes a D-galactosyl group accessible in immobilized pFn [16]. In addition to this carbohydrate-specific binding activity monocytes are known to express a receptor activity for fibronectin [17, 18] which was characterized [19, 20] as an integrin of the VLA family (VLA-5) with the possible additional presence of other integrin-like receptors [21]. These recognize, in a Ca^{2+}-dependent manner, an RGD-containing peptide sequence in the cell-adhesion domain of the fibronectin molecule.

In earlier experiments [22] we showed the cooperative action of carbohydrate-specific recognition via mCRP and peptide-specific binding via integrin-receptors in uptake of opsonized particles by rat macrophages. In continuation of this work we now present evidence that the same dual receptor recognition model may also hold true for the adhesion of human monocytes/macrophages to fibronectin-coated substratum.

Materials and methods

Materials Horseradish peroxidase conjugated rabbit anti-mouse IgG+IgM+IgA antibody was from Zymed (San Francisco, USA); Anti-CD14 mouse antibody was from Immunotech (Marseille, France) and Ficoll-Hypaque and

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Gelatin-sepharose column (20 ml bed size) were from Pharmacia (Uppsala, Sweden). Eagles' Medium and RPMI 1640 were from Gibco (Eggstein, FRG). Basal Medium Supplement (BMS) was from Biochrom (Berlin, FRG); Aurion-C-BSA from Aurion (Wageningen, NL); Blocking reagent, DIG Nucleic Acid Detection Kit and pentapeptide GRGDS were from Boehringer, Mannheim (FRG). Merckoglas and Sodium citrate were from Merck (Darmstadt, FRG). Lactosylated BSA was from Janssen (Beerse, Belgium) and mono- and disaccharides were from Fluka (Neu-Ulm, FRG). Multitest glass slides were from ICN Flow (Meckenheim, FRG).

Isolation of human blood monocytes Heparinized blood samples obtained from normal donors were used to prepare human blood monocytes by centrifugation over Ficoll-Hypaque [23]. For adhesion experiments, monocytes were adjusted to 4×10^6 monocytes per ml Eagle's Medium. The non-phagocytic cells were removed by adhesion on glass slides for 30 min in the incubator (37°C, 5% CO_2). Cells (50 000 monocytes per well) were rinsed with RPMI 1640 and cultured for 24 h in RPMI 1640 supplemented with 10% BMS.

Antibody labelling of isolated monocytes For antibody labelling, cells were fixed with acetone for 10 min on ice and washed in Tris-buffered saline (TBS A: 100 mM Tris-HCl, pH 7.6 containing 150 mM NaCl). Unspecific binding sites were blocked by a 30 min incubation with 0.2% cationized bovine serum albumin (Aurion-C-BSA) in TBS A, pH 7.6, followed by incubation with the neoCRP-specific monoclonal mouse antibody CrIs4 (11, 13) (dilution, 1:4) in TBS A plus 0.05% Aurion-C-BSA for 2 h or with monoclonal antibody against CD14 (1:200). As second antibody horseradish peroxidase conjugated rabbit anti-mouse IgG+IgM+IgA adsorbed against human serum was used at a final dilution of 1:50. Cells were rinsed three times in TBS A Buffer and then incubated at room temperature (RT) with 0.05% diaminobenzidine, 0.015% hydrogen peroxide for 10 min, dehydrated and embedded for light microscopy.

Preparation of RNA probes A plasmid vector pBr322 containing a human 2.2 kb CRP DNA fragment (pCRP5) [24] and a chicken 2.0 kb β-actin DNA-clone [25] as control were used for *in vitro* transcription of DNA as described previously [12]. RNA probes were labelled with digoxigenin-UTP by *in vitro* transcription according to the manufacturer's recommendations.

In situ *hybridization* After removal of medium, cells were dried quickly in a cold air stream, fixed for 2 h with 4% paraformaldehyde, 5% formaldehyde in phosphate-buffered saline (PBS), pH 7.0 at 4°C, dehydrated in a graded ethanol series and stored.

After rehydration the cells were washed in 100 ml of PBS and 0.02% Triton X-100 followed by 100 ml 2 × SSC (standard saline citrate: 1 × SSC = 0.015 M sodium chloride and 0.015 M sodium citrate), for 10 min each at room temperature (RT).

For prehybridization cells were incubated in hybridization buffer (deionized 50% formamide, 5% blocking reagent, 5 × SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS and 0.1 M Tris, pH 7.4) for 1 h at 4°C.

Cells were then hybridized with 25 ng of the digoxigenin-labelled RNA probe in 50 µl hybridization buffer. As controls, CRP sense and actin antisense RNA (labelled with the same specific activity) were used. Solutions were heated to 95°C for 10 min and chilled on ice prior to use. Hybridization was at 40°C in a moist chamber for 20 h.

Slides were rinsed briefly in 100 ml of PBS, 0.02% Triton X-100 for 5 min and 100 ml of 2 × SSC at RT for 10 min, in 100 ml of 1 × SSC , 0.01% SDS (twice, 10 min, at 50°C) and 100 ml of 0.5 × SSC, 0.01% SDS (10 min, RT).

Detection of RNA-RNA hybrids After hybridization, slides were incubated 30 min at RT with 0.2% Aurion-C-BSA, 0.3% Triton X-100 in TBS A, pH 7.6 (100 mM Tris-HCl, 150 mM NaCl) to block unspecific binding sites and incubated overnight at 4°C with anti-digoxigenin-antibodies conjugated with alkaline phosphatase (dilution, 1:1000) in 0.1% Aurion-C-BSA, 0.02% Triton X-100, TBS A, pH 7.6. Free antibodies were removed by rinsing in TBS A, pH 7.6. Then cells were equilibrated in TBS B (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min, and incubated with staining solution (337 µg ml^{-1} nitroblue tetrazolium salt, 175 µg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in TBS B, pH 9.5) for 4–20 h in the dark. Reaction was stopped by washing the slides for 5 min with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. For light microscopy cells were dehydrated in 2-propanol, cleared with xylene and embedded in Merckoglas.

Adsorption of LacBSA on to colloidal gold particles Colloidal golf particles 17 nm in diameter were prepared following the routine procedure of reducing a chloroauric acid solution with sodium citrate. Lactosylated BSA (LacBSA) was adsorbed onto the gold particles as described previously [26]. Gold particles were centrifuged, washed twice, resuspended in PBS-buffer and 0.05% PEG, and used immediately.

Uptake of LacBSA gold particles by adherent monocytes Human monocytes, after 24 h of culture, were preincubated with or without saccharides (80 mM final concentration) for 5 min prior to the addition of the ligand at 37°C. LacBSA gold particles (LacBSA-Au_{17}) were added at a final concentration of about 10^{12} particles per ml and cells were incubated for 1 h at 37°C. After incubation the cells were rinsed and fixed in acetone for 10 min on ice and prepared for light microscopy.

Plasma fibronectin (pFn) isolation Fibronectin was isolated as described [27]: 20 ml of plasma (with 10 mM EDTA) was applied to a gelatin-Sepharose column (20 ml bed size) and washed with TBS buffer C (20 mM Tris, 100 mM Nacl,

0.1 mM phenylmethylsulfonyl-fluoride). A second wash with TBS C plus 1 M urea was followed by elution of pFn with 4 M urea in TBS C. Purity of each isolation was checked by SDS-PAGE and silver staining [22]. Purified pFn was used after extensive dialysis against TBS C. The final concentration of fibronectin expressed as fibronectin monomer was 6 μM (1.2 mg ml^{-1}).

Substrate coating Fibronectin (20 μl of a 1.2 mg ml^{-1} solution in TBS C) was added to multitest glass slides undiluted or at the concentrations indicated. Absorption took place overnight in an incubator at 37°C.

Cell adhesion assay Isolated monocytes in Eagle's medium were incubated on precoated slides (2×10^5 cells per well) for 5–60 min at 37°C. For inhibition experiments cells were preincubated with mono- or disaccharides or with pentapeptide GRGDS (Gly-Arg-Gly-Asp-Ser) for 5 min. At the end of the incubation period, the slides were gently washed and attached cells were fixed with acetone. Cell adhesion was quantified by staining cells with anti-CD14. Positive cells were counted on five statistically distributed areas per well under the light microscope.

Results

Synthesis and expression of mCRP on human monocytes A monoclonal antibody (mAb), raised against rat mCRP and known to cross-react with human neoCRP was used for detection of mCRP in human monocytes. Expression of mCRP in freshly isolated monocyte suspension was found in 40.2% ± 33% (n = 9) of cells. An increase in positivity was observed after 1 h of culture (63.8% ± 31.4; n = 4) and the same was found after a 24 h culture period (67.5% ± 30%, n = 13; Fig. 1a and b). RNA-RNA *in situ* hybridization using a human CRP cDNA clone was used to search for the presence of a CRP probe in isolated human monocytes. Hybridization with antisense CRP RNA stained the same major subpopulation (63.8% ± 18%, n = 25) of human monocytes cultured for 24 h (Fig. 1c). As controls, actin-specific RNA stained 100% of cells and sense CRP RNA gave no signal at all (Fig. 1d). The large statistical errors are due to seasonal variations and variations between donors. However, the correlation between the data obtained by antibody labelling and *in situ* hybridization was excellent for a single cell preparation.

Endocytosis of colloidal LacBSA gold particles In cells cultured for 24 h, endocytosis of particles exposing terminal galactosyl groups (LacBSA-Au_{17}) was monitored by light microscopy. Accumulation of endocytosed particles (45.8% ± 22.5%) was recognized as a dark (red) stain within the cell cytoplasma (Fig. 1e). Uptake was completely blocked in the presence of *N*-acetyl-galactosamine (Fig. 1f), with 2.8% ± 2.8% positive monocytes ($p \geqslant 0.0003$).

Monocyte adhesion on immobilized fibronectin To assess the possible biological function of mCRP in monocyte adhesion we tested for carbohydrate-specific cell attachment on fibronectin-coated substrate. Cell adhesion was found to depend on the fibronection concentration used for coating (Fig. 2). Maximal cell adhesion was found at a fibronectin concentration of 6 μM (92 ± 11.4%) and therefore all following experiments were performed at this plasma fibronectin concentration. Kinetics in the presence of either saccharose or lactulose (100 mM each) are shown in Fig. 3. Half maximal adhesion is found within 15 min in the presence of saccharose and maximum cell attachment is reached 25 min after seeding. In the presence of lactulose cell attachment was blocked and did not exceed an average of 8.3 cells per unit area even after 60 min.

Inhibition by lactulose was concentration dependent, with half-maximal inhibition achieved at a concentration of 8 mM (Fig. 4). Adhesion was clearly a galactose-specific event as only addition of carbohydrates with galactosyl-residues resulted in inhibition (Fig. 5A and B).

In accord with previous observations addition of the pentapeptide GRGDS also led to a significant and concentration-dependent inhibition of adhesion (Fig. 6A).

Interestingly, and in contrast to our earlier findings with rat liver macrophages, we found no synergistic effect when using submaximal doses of both lactulose and GRGDS (Fig. 6B). Rather, simultaneous incubation with pentapeptide and lactulose led to an inhibition identical to the one seen with respective lactulose concentrations alone, which is less inhibitory than values for pentapeptide at 0.5 mg ml^{-1} (Fig. 6A). Chelation of calcium by EDTA led to complete inhibition in accord with the known calcium-dependence of both receptor activities (Fig. 6C).

Discussion

C-reactive protein (CRP) is an acute phase marker belonging to the pentraxin family. In the results presented previously we demonstrated that mCRP on rat liver macrophages (Kupffer cells) is identical with a galactose-specific particle receptor activity on their surface [11] and is synthesized within these cells [12]. By RNA-RNA *in situ* hybridization experiments we now find CRP-specific mRNA expression in about 70% of human monocytes; the presence of mCRP is found in about the same percentage of cells using indirect immunolabelling with a monoclonal antibody previously shown to recognize a neoCRP epitope [11].

We suggest that the biological function of mCRP on human monocytes may be similar to that of the selectins, for which low affinity carbohydrate-specific binding activities have been shown to be involved in lymphocyte adhesion to endothelial cells as an initial event in tissue invasion or lymph node homing. Our adhesion experiments demonstrate that a similar phenomenon may be operative in human monocyte/ macrophage infiltration or homing. We find that cell attachment to a fibronectin

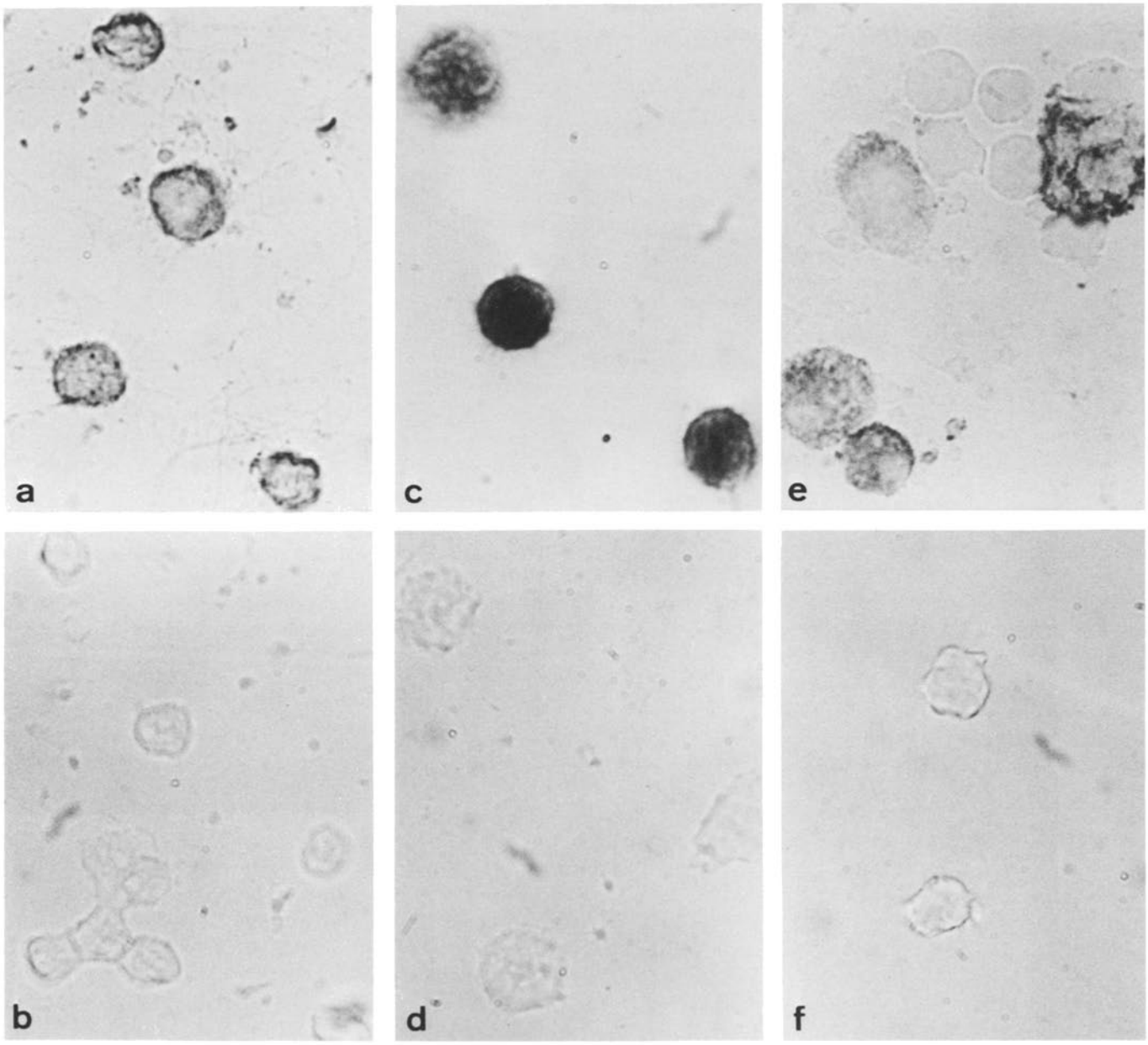

Figure 1. Expression and synthesis of mCRP in isolated human monocytes cultured for 24 h. Indirect immunocytochemistry of monocytes with anti-neoCRP specific antibodies (a) reveals string positive staining. Controls using labelled second antibody only (b) were negative. RNA-RNA *in situ* hybridization of monocytes with antisense human CRP-derived RNA gave a strong positive signal (c). No staining was found with the sense RNA (d). Monocyte endocytosis of gold particles opsonized with LacBSA (LacBSA-Au_{17}) is recognizable by the dark (red) patchy staining of cells (e). Monocytes, preincubated with *N*-acetyl-galactosamine (80 mM) for 5 min prior to addition of LacBSA-Au_{17}, showed no intracellular accumulation of stain (f). (Magnifications × 1500).

layer is a carbohydrate-specific event, with specificities identical to previously published [28] reports for CRP-fibronectin interactions, suggesting that mCRP may be responsible for the carbohydrate-dependent adhesion. As fibronectin is a known ligand for a number of integrin receptors [29], we also searched for peptide-specific binding activity. Indeed, the pentapeptide Gly-Arg-Asp-Ser also blocked monocyte attachment as described earlier [18]. Interestingly, combinations of the two inhibitors did not lead to synergistic blockade of adhesion. The presence of lactulose apparently abrogated the inhibitory effect of the pentapeptide as the relative degree of inhibition was always identical with that achieved by the disaccharide alone, which is significantly less than the inhibition achieved with pentapeptide alone. These results are consistent with what is already known about selectin-mediated adhesion events, where the lectin-like binding is an initial event which serves as a prerequisite for the

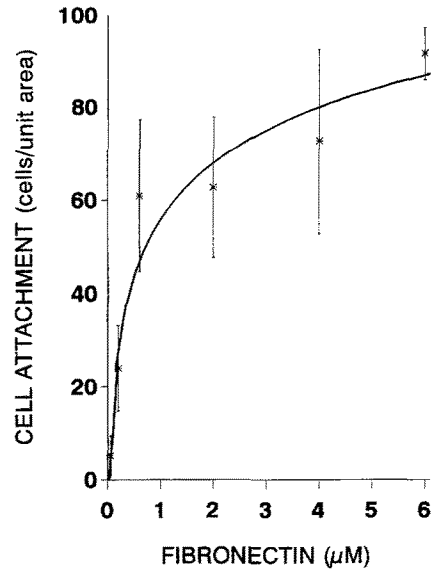

Figure 2. Monocyte attachment on fibronectin-coated surfaces. Cells were seeded on to glass slides preincubated with various concentrations of fibronectin (0.06–6 μM). The number of cells attached per unit area was found to depend on the concentration of fibronectin present in the preincubation step. Data are the mean of three experiments ± SEM.

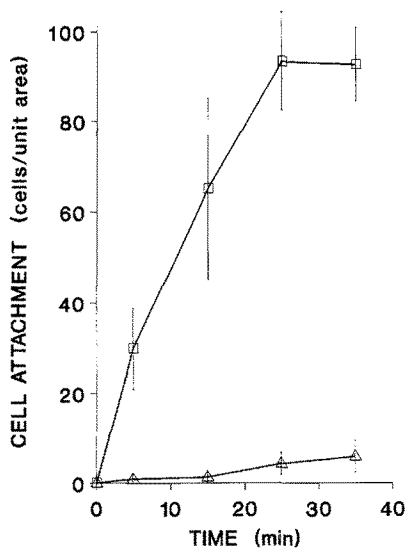

Figure 3. Kinetics of cell attachment of fibronectin-coated surfaces. Cells were pretreated with 100 mM saccharose (open squares) or with 100 mM lactulose (open triangles), seeded on substrates coated with fibronectin, and quantified after various time intervals. Cell attachment in the presence of saccharose is completed within 25 min. The inhibition by lactulose lasted throughout the experiments. Data are the mean of three experiments ± SEM.

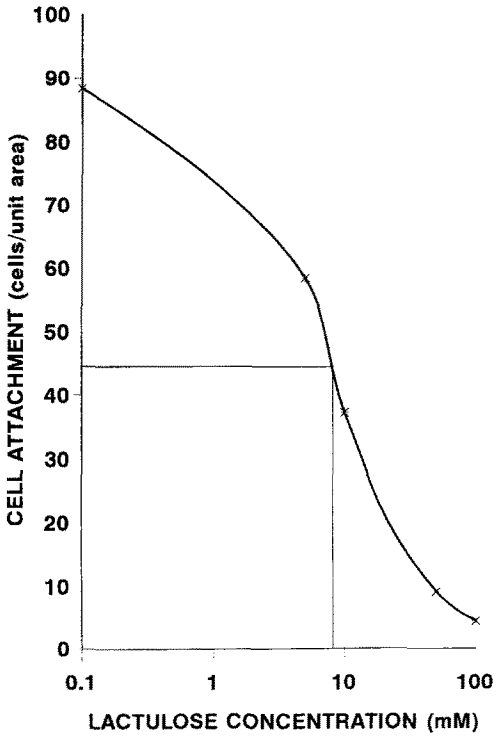

Figure 4. Concentration-dependent inhibition of cell attachment to fibronectin by lactulose. Lactulose showed a significant inhibitory effect, dependent on its concentration, with a half maximal inhibition at 8 mM. Data are the mean of three experiments ± SEM.

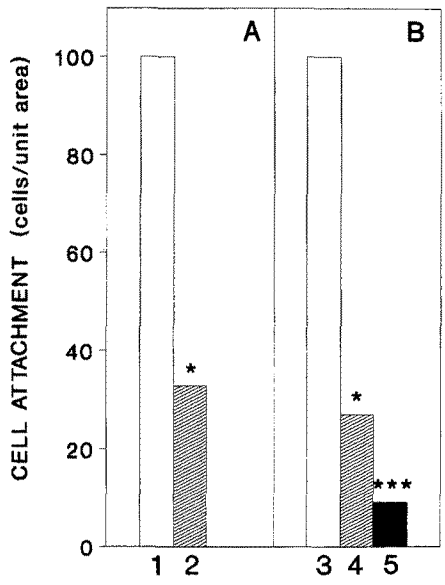

Figure 5. Cell attachment inhibition by mono- and disaccharides. Isolated monocytes were preincubated with various monosaccharides (50 mM) for 5 min (A), prior to seeding on fibronectin-coated substrates for 25 min and measurement of attachment. Cell adhesion was inhibited by galactose (bar 2) but not by glucose (bar 1). Significance was $p \leq 0.04$. Lactose (bar 4) and lactulose (bar 5) but no saccharose (bar 3) led to a significant reduction in cell adhesion (B). The data represent the mean of three experiments ± SEM. Significance was $p \leq 0.04$ (*) for lactose inhibition and $p \leq 0.00001$ for lactulose.

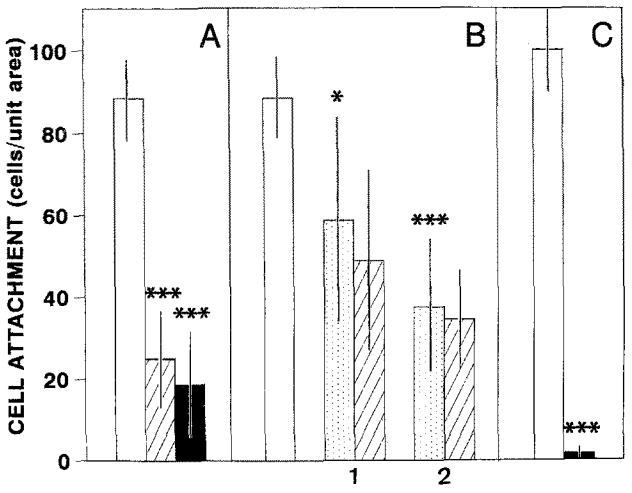

Figure 6. Inhibition of cell attachment by pentapeptide GRGDS alone or in combination with lactulose or by EDTA. Monocytes were pretreated with GRGDS (6A) at two different concentrations (0.05 mg ml^{-1} striped bar and 1.0 mg ml^{-1} closed bar), before seeding on fibronectin-coated substrates. GRGDS exerts significant inhibition compared with the control (open bar). Simultaneous incubation (B) with pentapeptide (0.5 mg ml^{-1}) and lactulose (hatched bars) led to an inhibition with no significant difference to the one seen with the respective lactulose concentration alone (dotted bars). Incubation in (1) was in the presence of 5 mM lactulose and in (2) in the presence of 10 mM lactulose. EDTA (6mM) results in complete inhibition of cell adhesion (C: control, clear bar; EDTA, dark bar). Attached cells were always quantified 25 min after seeding by counting CD14 positive cells. The significance, compared with the controls, was $p \leq 0.004$ (*) or $p \leq 0.00001$ (***). Data represent the mean of three experiments ± SEM.

integrin-mediated stable binding as the second event. Indeed, our experiments with inhibitor combinations demonstrate that the same sequence of events may also be true for monocyte adherence to fibronectin-coated surfaces. Apparently the carbohydrate-specific binding is a prerequisite for integrin binding since in the presence of inhibitory saccharides no pentapeptide-blockable interactions occur. The remaining binding activity found in the presence of competing disaccharide appears to involve a third receptor-ligand interaction not yet identified. This interaction is neither galactose-specific nor involves RGD recognition. In conclusion, we show for the first time that mCRP is synthesized and expressed in human monocytes and may act as a selectin-like molecule involved in monocyte adherence to immobilized fibronectin. Since fibronectin is a major component of the extracellular matrix and ubiquitously present on cell surfaces, these experiments represent a model for the extravasation and tissue infiltration of monocyte-macrophages.

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References

1. Kolb-Bachofen V (1991) *Immunobiology* **183**:133–45.
2. Volanakis JE, Kaplan MD (1971) *Proc Soc Exp Med Biol* **136**: 612–14.
3. Anderson JK, Stroud RM, Volanakis JE (1978) *Fed Proc* **37**:1495.
4. Kaplan MH, Volanakis JE (1974) *J Immunol* **112**:2135–47.
5. Salonen EM, Vartio T, Hedman K, Vaheri A (1984) *J Biol Chem* **259**:1496–1501.
6. Tseng J, Mortensen RF (1988) *Mol Immunol* **25**:679–86.
7. Robey FA, Jones KD, Tanaka T, Liu TY (1984) *J Biol Chem* **259**:7311–16.
8. Du Clos TW, Zlock LT, Rubin RL (1988) *J Immunol* **141**: 4266–70.
9. Higginbotham JD, Heidelberger M, Gotschlich EC (1970) *PNAS* **67**:138–42.
10. Emsley J, White HE, O'Hara BP, Oliva G, Srinivasan N, Tickle IJ, Blundell TL, Pepys MB, Wood SP (1994) *Nature* **367**:338–45.
11. Kempka G, Roos RH, Kolb-Bachofen V (1990) *J Immunol* **144**:1004–9.
12. Egenhofer C, Alsdrorff K, Fehsel K, Kolb-Bachofen V (1993) *J Hepatology* **18**:1216–23.
13. Potempa LA, Siegel JN, Fiedel BA, Potempa RT, Gewurz H (1987) *Mol Immunol* **24**:531–41.
14. Zahedi K, Tebo JM, Siripont J, Klimo F, Mortensen RF (1989) *Immunol* **142**:2384–92.
15. Eallou SP, Buniel J, Macintyre SS (1989) *J Immunol* **142**: 2708–15.
16. Kolb-Bachofen V, Abel F (1991) *Carbohydr Res* **214**:201–13.
17. Villinger B, Kelley DG, Engleman W, Kuhn C, McDonald JA (1981) *J Cell Biol* **90**:711–20.
18. Hosein B, Bianca C (1985) *J Exp Med* **162**:157–70.
19. Argraves WS, Suzuki S, Thompson K, Pierschbacher MD, Rouslahti E (1987) *J Cell Biol* **105**:1183–90.
20. Holers VM, Ruff TG, Parks DL, McDonald JA, Balliard LL, Brown EJ (1989) *J Exp Med* **169**: 1589–605.
21. Gresham HD, Goodwin JL, Allen PM, Anderson DC, Brown EJ (1989) *J Cell Biol* **108**:1935–43.
22. Kolb-Bachofen V (1992) *J Clin Invest* **90**:1819–24.
23. Boyum A (1968) *J Clin Lab Invest* **Suppl 21**:77–89.
24. Woo P, Korenberg JR, Whitehead AS (1985) *J Biol Chem* **260**:13384–88.
25. Cleveland DW, Lopata MA, McDonald RJ, Cowan NK, Rutter WJ, Kirschner MW (1980) *Cell* **20**:95–105.
26. Kolb-Bachofen V (1989) *In Methods in Enzymology* (Ginsburg V, ed.) Vol 179. pp. 111–21. San Diego: Academic Press Limited.
27. Engvall E, Ruoslahti E (1977) *Int J Cancer* **20**:1–5.
28. Köttgen E, Hell B, Kage A, Tauber R (1992) *J Immunol* **149**:445–53.
29. Rouslahti E (1988) *Ann Rev Biochem* **57**:375–413.