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Development of the Phage Displayed Peptide as an Inhibitor of MCP-1 (Monocyte Chemoattractant Protein-1)-mediated Angiogenesis

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Abstract

The CC chemokine, monocyte chemoattractant protein-1 (MCP-1), plays a crucial role in the initiation of atherosclerosis and has direct effects that promote angiogenesis. To develop a specific inhibitor for MCP-1-induced angiogenesis, we performed in vitro selection employing phage display random peptide libraries. Most of the selected peptides were found to be homologous to the second extracellular loops of CCR2 and CCR3. We synthesized the peptide encoding the homologous sequences of the receptors and tested its effect on the MCP-1 induced angiogenesis. Surface Plasmon Resonance measurements demonstrated specific binding of the peptide to MCP-1 but not to the other homologous protein, MCP-3. Flow cytometry revealed that the peptide inhibited the MCP-1 binding to THP-1 monocytes. Moreover, CAM and rat aortic ring assays showed that the peptide inhibited MCP-1 induced angiogenesis. Our observations indicate that the MCP-1-binding peptide exerts its anti-angiogenic effect by interfering with the interaction between MCP-1 and its receptor.

Introduction

Chemokines are inflammatory cytokines that play critical roles in leukocyte recruitment during several pathological processes. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine implicated in a variety of inflammatory diseases, such as atherosclerosis and rheumatoid arthritis. The recruitment of monocytes into the arterial subendothelium is one of the earliest steps in the development of atherosclerosis, and MCP-1 plays a crucial initiating role in this process. MCP-1 is not expressed in normal endothelium, but it is induced by diverse stimuli such as cytokines and oxidized adducts, and secreted by endothelial cells, smooth muscle and macrophages.

Secreted MCP-1 binds to CCR2 (CC chemokine receptor 2) with high affinity and induces leukocyte chemotaxis. The extracellular amino terminal domain of CCR2 (amino acids 2-36) is critical for initiating binding, and one or more of its extracellular loops may also be involved in binding.

MCP-1 selectively binds to its cognate receptor CCR2; it also binds to the Eotaxin receptor CCR3 with low affinity, but its biological significance is not currently understood. Eotaxin is a natural antagonist of MCP-1, presumably because it inhibits the MCP-1/CCR2 interaction. The basis of selective binding between chemokines and their receptors is not clearly understood and is the subject of current investigation.

Chemokines also regulate angiogenesis directly, as a consequence of leukocyte infiltration, or growth factor expression. MCP-1 is the first CC chemokine reported to play a direct role in tumor angiogenesis, and eotaxin also induces in vivo angiogenic responses by using endothelial cells expressing CCR3. Even though the details of chemokine-induced angiogenesis are not fully understood, therapy based on inhibiting the angiogenic function of MCP-1 could be of value for inhibiting tumor progression and preventing the development of atherosclerosis.

Phage libraries display random peptides as the coat protein fusions on the surface of bacteriophage particles. They permit rapid identification of peptide ligands that contain motifs for interacting with a target molecule, and may act as inhibitors of that target. We used a phage display peptide library to isolate peptides that bind to MCP-1. Interestingly, most of the isolated peptides were homologous to the second extracellular loops of CCR2 and CCR3, which were previously implicated to be involved in MCP-1 mediated signal transduction. Most significantly, CAM and rat aortic ring assays revealed that the second extracellular loop-mimetic peptide inhibited MCP-1-induced angiogenesis. The selected peptide could be useful as a lead molecule for anti-atherosclerosis therapy.

Results

In vitro selection and synthesis of MCP-1 binding peptides

To select peptide ligands to MCP-1 we used two libraries (Ph.D-7 and Ph.D-12) with peptides of 7 and 12 random amino-acids, respectively, displayed on the pIII coat protein of phage M13. Recombinant human MCP-1 protein was the target for the phage selection. A significant increase in relative phage yield was obtained after 3 rounds of biopanning. Of the 27 clones from the Ph.D.-7 library, thirteen had identical sequences (designated 7-1) and the others were similar but not identical. When we compared the peptide sequences to whole sequences of CCR2 and CCR3, most of the selected peptide sequences were homologous to second extracellular loops between transmembranes 4 (TM4) and 5 (TM5). In particular, the peptide sequences were similar to the portion of the loop (amino acids 198-204 of CCR2 and 195-201 of CCR3) which was also conserved between CCR2 (WNNFHTI) and CCR3 (WRHFHTL). Since the most frequently found peptide 7-1 was similar to the homologous sequences of CCR3 (WRH), we synthesized peptide #1 (HSWRHFHTLGGG) containing CCR3 amino acids 195-201 (underlined) and flanking sequences from M13 coat protein. As a control, we also synthesized peptide #1 mutant (HSARHAHTLGGG).

Binding of peptides to MCP-1

To quantify the binding affinity of the peptide aptamer, we used Surface Plasmon Resonance (SPR) technology. An example of an SPR sensorgram to MCP-1 protein is shown. When the same peptide concentrations were injected, the binding profile of peptide #1 (#1) was much more pronounced than that of the #1 mutant (#1 mut.) or the negative control (NC) peptide (SHWDQPRPGLKP) that binds to sialyl Lewis X carbohydrates. To confirm the binding specificity of the peptide #1 to MCP-1, the SPR profiles of peptide #1 and #1 mutant were examined for the binding to the homologous protein, MCP-3 (72% sequence identity to MCP-1). It was clearly demonstrated that the MCP-1 binding peptide did not bind even to such a homologous chemokine. By injecting various concentrations of the peptides under kinetic conditions, we obtained the dissociation constants of peptide #1 to MCP-1 protein. The binding affinity of peptide #1 to MCP-1 was $2.20 \pm 0.44 \times 10^{-5}$ M, which was extremely high affinity for such a small size peptide evaluated by SPR analysis. It was not appropriate to define the binding affinities for control peptides binding to MCP-1 as well as for peptide #1 binding to MCP-3, because the binding profiles most likely resulted from non-specific binding.

Since peptide #1 bound to MCP-1 with high affinity in vitro, we tested whether it could inhibit MCP-1 binding to THP-1 cells. FACS analysis showed that peptide #1 inhibited the binding of biotin-MCP-1 to THP-1 cells up to 70% and the specificity of inhibition was confirmed by comparison with mutant #1 peptide and anti-MCP-1 antibody. Even though the THP-1 cell is known to express high levels of CCR2 on the cell surface, we do not know how the peptide inhibits the MCP-1 from binding to THP-1 cells. Whatever the mechanism, these results suggested that excess peptide #1 might compete with MCP-1 for binding to the surface of THP-1 cells.

Inhibition of angiogenesis by the peptide

Since peptide #1 bound to MCP-1, we tested whether it affected MCP-1-induced angiogenesis. MCP-1 stimulates the formation of robust new blood vessels, as shown by CAM assays. Application of excess amounts of peptide #1 to the MCP-1 mixture greatly reduced the number of micro-vessels formed, and this anti-angiogenic effect was specific, since neither #1 mutant nor NC peptides had a significant inhibitory effect. Quantification of the number of branch points revealed the specific inhibition by peptide #1. To investigate whether ex vivo angiogenesis was also stimulated by MCP-1, we used the rat aortic ring-sprouting assay. The control experiments demonstrated that MCP-1-induced angiogenesis was not affected by #1 mutant or NC peptides, whereas peptide #1 inhibited ring sprout formation in a dose-dependent manner. We estimated that the inhibitory concentration (IC_{50}) of peptide #1 was around 10 μM.