BINDING OF ENDOTHELIN-1 TO HUMAN BLOOD MONOCYTE

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ABSTRACT

Monocyte attachment to the endothelium and migration into the vessel intima are the initiating steps in atherogenesis. This is thought to be facilitated by endothelin-1 (ET-1) as a potent chemoattractant to human blood monocytes. To explore the presence of ET-1 receptor(s) on the monocyte, we studied the binding of ET-1 to freshly isolated human blood monocytes, in the laboratory of the Institute of Clinical Biochemistry, University of Bon, Germany in 1995. Radioligand binding studies revealed the presence of two distinct subclasses of binding sites with apparent dissociation constants, K_ds, of 10.3 pM and 3.5 nM and maximal binding capacities, B_{max}s, of 0.027 fmol and 0.63 fmol/1.5x10^5 cells. Using monocyte migration as a response to ET-1, and ET-1 receptor antagonists BQ-123, BQ-18257B and IRL-1038, the presence of two ET receptor subtypes, ET_A and ET_B, were detected. These results suggest that the chemotactic stimulus introduced by ET-1 can be activated ET-1 specific receptors on the monocytes.

Key words: Endothelin-1, monocyte, receptor-binding

IKATAN ENDOTELIN-1 PADA MONOSIT DARAH MANUSIA

ABSTRAK

Menempelnya monosit ke permukaan endotel dan bermigrasi kedalam tunika intima merupakan langkah awal pada aterogenesis. Hal ini diduga diperantarai oleh peran endotelin-1 (ET-1) yang dikenal sebagai *chemoattractant* poten bagi monosit. Untuk mengungkapkan adanya reseptor ET-1 pada monosit, dilakukan penelitian ikatan ET-1 pada monosit yang diisolasi dari darah manusia, di laboratorium Institute of Clinical Biochemistry, Universitas Bonn, Jerman pada tahun 1995. Penilaian ikatan *radioligand* menunjukkan adanya dua subkelas berbeda dari tempat ikatan dengan konstanta disosiasi (K_d) masing-masing 10,3 pM dan 3,5 nM, serta kapasitas ikatan maksimal (B_{max}) masing-masing sebesar 0,027 fmol dan 0,63 fmol/1,5x10^5 sel. Dari hasil penilaian tingkat migrasi monosit sebagai respons terhadap ET-1 dengan atau tanpa beberapa antagonis reseptor ET-1, BQ-123, BQ-18257B dan IRL-1038, terdeteksi adanya dua subtipe reseptor ET, yaitu ET_A dan ET_B. Hasil ini menunjukkan bahwa rangsangan kemotaksis yang ditimbulkan ET-1 dapat mengaktifkan reseptor spesifik ET-1 pada monosit.

Kata kunci: Endotelin-1, monosit, ikatan-reseptor

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INTRODUCTION

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide consisting of 21 amino acids; it was first isolated from cultured porcine endothelial cells.[1] Since this initial discovery, cells derived from many different tissues have been found to synthesize and secrete the peptide.[2-3] Subsequently, the cloning and sequencing of ET related genes revealed the existence of two additional peptides, ET-2 and ET-3.[4] ETs have variety of biological actions and have been implicated in the pathogenesis of many diseases, such as atherosclerosis.[5,6] In the latter disease, monocyte attachment to the endothelium and migration into the vessel intima are the initiating steps in atherogenesis.[7] We found that ET-1 is a strong chemoattractant to blood monocytes,[8] supportive of the role of ET-1 in the pathogenesis of atherosclerosis. Little information is known regarding the presence of ET receptor(s) in monocyte. ETs initiate cellular effects and physiological actions after binding to cell surface membrane receptors. Changes in intracellular Ca^{2+}, c-AMP, c-GMP, and activation of protein kinases are some of the responses subsequent to ligand-receptors, which is still to be elucidated in monocytes. The present study was designed to explore the occurrence of binding sites for ET-1 on human blood monocytes and to study the effect of receptor antagonists on monocyte migration.

MATERIALS AND METHODS

The materials used were obtained from the following sources: twenty four-well plates (Cat.No. 3047) and cell culture insert (Cat.No.3095) from Becton Dickinson, Heidelberg; NycoPrep 1068 and NycoPrep 1063 from Nycomed Pharma, Oslo, Norway; human serum albumin from Behringwerke AG, Marburg; Dextran T-500 from Biozym Diagnostik, Hameln. Mayer's hemalum solution from Merck, Darmstadt; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) from Serva, Heidelberg; fetal calf serum and Medium RPMI 1640 from Biochrom, Berlin; Endothelin-1 human (synthetic) and penicillin-streptomycin from Sigma, ^{125}I-ET-1 (specific radioactivity 2000 Ci/mmol) from Amersham and endothelin receptor antagonist BQ-123, BE-18257B and IRL-1038 from Alexis Corporation, Switzerland.

Monocyte Isolation

Human monocytes were isolated from freshly drawn blood in EDTA (1.6 mg/mL) Monovettes (Sarstedt, Numrecht); details have been described previously.[8]

Binding Assay

The binding assay was done in the laboratory of Institute of Clinical Biochemistry, University of Bonn, Germany, in 1995. To determine the binding of ^{125}I-ET-1 to monocytes as a function of time, freshly isolated monocytes (150,000 cells/100 µL) were incubated for various lengths of time with ^{125}I-ET-1 (3×10^{10}M) in the absence or in the presence of 1 µM of unlabeled ET-1; the total volume was 200 µL and the temperature was 37^{0}C. The microcentrifuge tube (capacity 400 µL, Beckman, Munich) containing the incubation contents were on a layer of a mixture of 100 µL of silicone oil (Silicon oil AR 20: AR 200/1:1, v/v). The incubation was terminated by centrifugation of the tubes at 10,000 g for 10s. The monocytes formed a pellet under the silicone oil layer. The tubes were cut at the oil level and the pellet was taken for measurement of radioactivity in a gamma counter (Packard, Cobra Auto-Gamma); the counting efficiency was 74%. Dependence of binding on the concentration of ^{125}I-ET-1, in the absence and in the presence of 1 µM of unlabeled ET-1, was determined as described above. Displacement of the radioligand from its binding sites was evaluated by incubating the monocytes with a constant concentration of ^{125}I-ET-1 (5×10^{10}M) and increasing concentrations of unlabeled ET-1 and processed as mentioned above. Specific binding was defined as total binding minus non-specific binding that occurred in the presence of an excess (1 µM) of unlabeled ET-1. The dissociation constant, K*d*, and the maximal binding capacity, B*max*, were determined using the Ligand program developed by Munson and Rodbard.[9]

Chemotaxis assay

Chemotaxis of monocytes was assayed by the method described previously[8] in a 24-well-plate with cell culture inserts. The bottom wells (500 µL) contained various concentrations of ET-1 dissolved in RPMI 1640 medium. Monocytes were added to the upper wells (cell culture insert) in a volume of 250 µL which contained 1.25×10^5 cells per well. After 60 min of incubation at 37^{0}C in a humidified incubator with air and 5% CO_2, the upper wells were removed and the non-migrated cells, i.e., the cells that did not settle on the membrane-bottom of the well, were removed gently by sucking up with a pipette. Cells on the membrane were carefully washed with PBS to remove the rest of the cells which were not firmly attached. They were then fixed with methanol and stained with Mayer's hemalum solution for 7 min. The cells firmly attached to the membrane were counted with a 12.5-fold-ocular and a 10-fold-objective with four counting grids. Five areas containing 4 grids were counted per well and

averaged. The mean variation among the counted field was 12 ± 6% for the test solutions and 19 ± 8% for the controls. Chemotaxis activity is expressed as chemotactic index, CI, defined as the ratio of the number of cells migrating in the response to ET-1 to the number of cells migrating when only medium was in the lower chamber, which was also the control. Receptor subtype was evaluated by incubating the monocytes with specific endothelin receptor antagonists BQ-123 (0.1 and 10 μM), BE-18257B (1 μM) or IRL-1038 (1 μM) for 60 min followed by determination of chemotaxis toward ET-1 as described above.

RESULTS

The binding of 125I-ET-1 to monocytes was time dependent; it increased progressively up to 60 min and reached an apparent steady state thereafter (Fig. 1); non-specific binding varied between 30% and 50% of total binding over the time range.

Binding of ^{125}I-ET-1 increased with increasing concentrations of ^{125}I-ET-1, the kinetics of which appeared to deviate from the hyperbolic form of a typical concentration curve (Fig. 2). Analysis of the binding data by the Ligand program (Fig. 2 inset) revealed the presence of two distinct subclasses of binding sites. One had a high affinity and low binding capacity for ET-1, the apparent K*d* was 10.3 pM and the B*max* 0.027 fmol/1.5 x 10^5 cells. The second subclass of binding site had low affinity and high binding capacity for ET-1; the apparent K*d* and B*max* values were 3.5 nM and 0.63 fmol/1.5 x 10^5 cells, respectively.

Unlabeled ET-1 competitively displaced ^{125}I-ET-1 from its binding sites on the monocyte; the calculated inhibitor constants, K*i*s, [(EC_{50}/(1 + L/K)] for the two binding sites, calculated by the Ligand program, were 70 pM and 3.6 nM, respectively (Fig. 3).

Chemotaxis of monocytes toward varying concentrations, 10^{-10}–10^{-6}M, of ET-1 and in the presence of 2 fixed concentrations of the specific ET_A receptor antagonist, BQ-123, decreased migration (Fig. 4). At 0.1 μM of BQ-123 the decrease in CI ranged from 22% to 29%, at 10 μM the decrease ranged from 42% to 54%. Another ET_A receptor antagonist, BE-18257B (1 μM), also inhibited monocyte migration; the decrease in CI ranged from 20% to 35%. The ET_B receptor antagonist IRL-1038 (1 μM) reduced migration; the decrease in the CI ranged from 8% to 28% (Fig. 5).

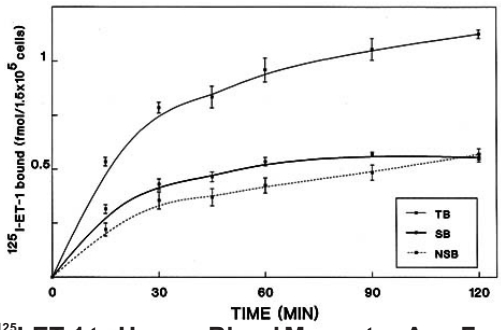

Figure 1 Specific Binding of ^{125}I-ET-1 to Human Blood Monoctye As a Function of Time

Freshly isolated monoctyes were incubated with 3 x 10^{-10} M ^{125}I-ET-1 at 37^{0}C for the times indicated. Specific binding (SB) was obtained by substracting non-specific binding (NSB) that occurred in the presence of 1 μM unlabeled ET-1 from total binding (TB). Each point is the mean ± SD of triplicate experiments.

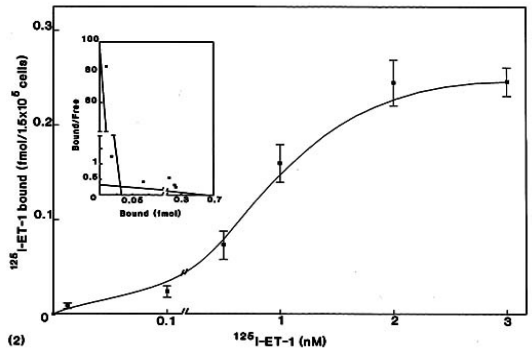

Figure 2 Saturable Binding of 125I-ET-1 to Human Blood Monocyte

Freshly isolated monocytes were incubated at 37^{0}C for 60 min with various concentration of ^{125}I-ET-1. Non-specific binding in the presence of 1 μM unlabeled ET-1 ranged from 30-50% of total binding. Each point is the mean ± SD of triplicate experiments. Inset shows the Scatchard plot of the binding data

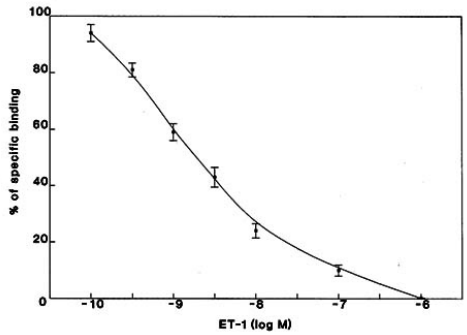

Figure 3 Displacement of ^{125}I-ET-1 from Its Binding Sites on Monocytes by Unlabeled ET-1

1.5 x 10^5 freshly isolated monoctyes were incubated with ^{125}I-ET-1 (5 x 10^{-10}M) in a total volume of 200 µl in the absence and presence of increasing concentrations of unlabeled ET-1. Each point is the mean ± SD of triplicate experiment.

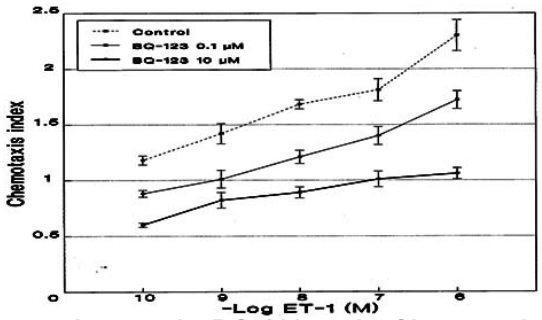

Figure 4 Influence of ET_A Receptor Antagonist BQ-123 on the Chemotaxis of Monocytes

The monocytes were pretreated with two different concentrations of BQ-123 for 60 min prior to the chemotaxis assay. The concentration range of ET-1 was 10^{-10}M-10^{6}M. The values are means and the vertical bars SDs from 3 separate experiments.

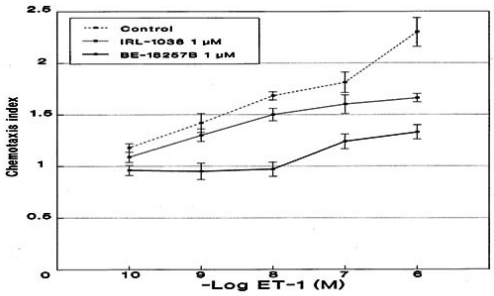

Figure 5 Influence of ET_A Receptor Antagonist BQ-1825B, and ET_B Receptor Antagonist IRL-1038 on the Chemotaxis of Monocytes

Treatment of monocytes with the receptor antagonists and the chemotaxis assay were performed as described in the legend for Fig.3. The values are means and the vertical bars

DISCUSSION

Monocyte attachment to the endothelium and migration into the intimal space of the blood vessel are construed as the earliest events in the chain of reactions leading to atherosclerotic changes of the vasculature. In patients with risk factors for atherosclerosis the circulating levels of ET-1 are increased.[10,11] Our previous study indicated that ET-1 possesses chemotactic activity for human blood monocytes. The underlying mechanism triggering migration was through Ca^{2+} influx since Ca^{2+} antagonists reduced migration.[8] In the present study we used ^{125}I-ET-1 to detect binding sites for ET-1 on the monocyte. The results clearly demonstrate, for the first time, the occurrence of two distinct subpopulations of binding sites for ET-1 on freshly isolated human blood monocytes. Recent studies have reported that at least two subtypes of binding sites for ET-1 isopeptides exist on the membrane endothelial cells.[12] The binding of ET-1 to blood monocytes was reversible and the affinity of the binding site with the low K*d* lies in the pM range agreeing with the concentration of ET-1 in plasma of normal individuals (0.1-7.98 pM).[13] The high affinity component possessed 108 sites and the low affinity component 2,529

sites per monocyte. The binding affinity of ET-1 to cells from different tissues[14,15] and the binding affinity of ET-1 to monocytes is almost similar, ranging from pM to nM. To date three ET receptors, ET_A, ET_B and ET_C, have been identified and several ET receptors antagonists have studied.[16-18] Using the chemotaxis as a response of monocytes to ET-1 and specific ET-1 receptor antagonists our study shows that at least two receptor subtypes are activated on the monocyte during ET-1 stimulus; one is the ET_A receptor which is affected by the antagonist BQ-123 and BE-18257 and the second one is the ET_B receptor which is affected by the antagonist IRL-1038. The consequence of receptor antagonism was decrease in the number of monocytes migrating in response to the chemotactic stimulus of ET-1. Apparently both receptors contribute to the chemotaxis process. Considering the affinities of the two receptor subtypes and the decrease in monocyte migration affected by the receptor antagonists, it is likely that the high affinity component is the ET_A receptor and the binding component with the low affinity the ET_B receptor, since ET-1 was bound with a higher affinity to the ET_A than to the ET_B receptor. The data also suggest that the ET_A receptors exercises a greater influence on chemotaxis than the ET_B recetors albeit their greater abundance. The different affinities and capacities of the endothelin receptors suggest that endothelin isopeptides are involved in a wide range of physiological and pathological events in an organism or cells, as well as in monocytes.[19] In conclusion, our data provide evidence for the occurrence of specific receptors for ET-1 on human blood monocytes. Redistribution or a change in directional orientation of these receptors by ET-1 probably enhances monocyte migration.

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