Targeting of Endothelin Receptors for Molecular Imaging of Atherosclerosis in Rabbits

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We wanted to determine whether a previously described in vivo accumulation of a ^{99m}Tc-labeled endothelin derivative in atherosclerotic lesions is mediated by binding to endothelin receptors. Furthermore, the expression of endothelin receptors in atherosclerotic lesions of 2 different rabbit animal models for atherosclerosis was to be evaluated to determine whether endothelin receptors generally are a suitable target for atherosclerosis imaging. **Methods:** Normal vessels from untreated New Zealand White rabbits (NZW), balloon-denuded aortas from cholesterol-fed NZW, and atherosclerotic aortas from Watanabe Heritable Hyperlipidemic rabbits (WHHL) were used either as cross sections (cryosections) for receptor binding studies or for superfusion with a medium containing ^{125}I-labeled endothelin-1 or the ^{99m}Tc-labeled endothelin derivative. **Results:** Cross sections of aortas from untreated NZW contained $45 \pm 11 \cdot 10^6$ endothelin A receptors per square millimeter and $55 \pm 11 \cdot 10^6 \cdot$ endothelin B receptors per square millimeter, cross sections of balloon-denuded aortas from cholesterol-fed NZW contained $106 \pm 16 \cdot 10^6$ endothelin A receptors per square millimeter and $27 \pm 16 \cdot 10^6$ endothelin B receptors per square millimeter, and cross sections of atherosclerotic aortas from WHHL contained $40 \pm 13 \cdot 10^6$ endothelin A receptors per square millimeter and $5 \pm 13 \cdot 10^6$ endothelin B receptors per square millimeter. Balloon-denuded aortas from cholesterol-fed NZW (366 ± 132 amol$\cdot$mm^{-2}, $P < 0.001$) and atherosclerotic aortas from WHHL (338 ± 175 amol$\cdot$mm^{-2}, $P < 0.002$) accumulated significantly more of the ^{99m}Tc-labeled endothelin derivative than did vessels from control animals (137 ± 26 amol$\cdot$mm^{-2}). On the contrary, ^{125}I-labeled endothelin-1–bound receptor mediated to superfused aortas from untreated NZW (12 ± 9 amol$\cdot$mm^{-2}) and to balloon-denuded aortas from cholesterol-fed NZW (19 ± 5 amol$\cdot$mm^{-2}) but not to aortas from WHHL. This lack of receptor-specific accumulation of ^{125}I-endothelin-1 in atherosclerotic areas of WHHL aortas, and this receptor-specific accumulation in atherosclerotic balloon-denuded NZW aortas that does not significantly increase in comparison with normal aortas of untreated NZW, cause failure of endothelins to detect atherosclerotic lesions. **Conclusion:** Although the density and the ratio of endothelin receptor subtypes change because of the development of atherosclerotic lesions in rabbit aortas, endothelin receptor targeting for imaging of atherosclerosis is not suitable.

Key Words: endothelin derivative; endothelin receptor subtypes; atherosclerosis; molecular imaging; perfusion apparatus; receptor targeting

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Since the first description of endothelins in 1988 (*1*), it has become evident that endothelins and their receptor subtypes A and B are involved in the development of atherosclerosis (*2–8*) and restenosis after balloon angioplasty with (*9*) or without (*10*) stent placement. Therefore, on the basis of published affinity studies (*11–13*), we developed an endothelin derivative (Asp-Gly-Gly-Cys-Gly-Cys-Phe-(D-Trp)-Leu-Asp-Ile-Ile-Trp) for whole-body scintigraphy. With this peptide, experimentally induced atherosclerotic lesions could be detected in vivo (*14*) in an animal model. The accumulation of this endothelin derivative correlated well with the amount of vascular smooth muscle cells in the neointima, but whether the accumulation was indeed mediated through binding of this endothelin derivative to endothelin receptors remained unclear. It still remains unknown whether the diagnostic properties of endothelin derivatives can be improved by enhancing the affinity and specificity to 1 of the 2 endothelin receptor subtypes. The differential expression of the endothelin receptor subtypes in atherosclerotic lesions in comparison with normal vessels remains controversial (*9,10,15–24*).

This study investigated which endothelin receptor subtype is expressed in atherosclerotic lesions of different animal models and whether the observed accumulation of the endothelin derivative is indeed mediated through endothelin receptor binding and, thus, whether this process is suited to detect atherosclerotic lesions by imaging endothelin receptors. In addition to duplicating previous work in which New Zealand White rabbits (NZW) underwent balloon denudation, this study was expanded by additionally investigating Watanabe Heritable Hyperlipidemic rabbits (WHHL). WHHL lack low-density lipoprotein receptors, and severe atherosclerosis spontaneously develops in them after several months (*25*).

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MATERIALS AND METHODS

Animals

The study used 20 female NZW (body weight range, 4.0–5.0 kg; Schriever, Bremervörde, Germany) and 11 female WHHL (body weight range, 4.5–6.0 kg; Emsicon-Jung, Vaterstetten, Germany). Nine NZW underwent balloon denudation as previously described (*14*) and additionally received a 0.5% cholesterol diet (Altromin, Lage, Germany) for 6 wk after intervention. Animal care complied with principles of good laboratory practice, and the study was approved by the Institutional Board for Laboratory Animal Care.

Preparation of Aortic Tissue for In Vitro Binding Studies

The animals were killed by intravenous injection of 2 mL T61 (Hoechst, Unterschleissheim, Germany). The aortas were excised and immediately frozen in liquid nitrogen. Cryosections with a thickness of 30 μm for competitive binding assays and a thickness of 10, 20, 30, 40, 60, and 90 μm for saturation binding assays were made using a microtome (Microm, Walldorf, Germany). Each cryosection was transferred to a single well of gelatin-coated 48-well plates. The plates were dried and stored at −20°C. For saturation binding assays, the 48-well plates were scanned with a computer scanner and the area of each cross section was determined (ImagePC; Scion Corp., Frederick, MD).

Determination of Endothelin Receptor Subtypes and Their Densities

Cryosections were rehydrated for 1 h with buffer (140 $mmol \cdot L^{-1}$ NaCl, 1 $mmol \cdot L^{-1}$ $MgCl_2$, 1.25 $mmol \cdot L^{-1}$ $CaCl_2$, 4 $mmol \cdot L^{-1}$ KCl, 1 $mmol \cdot L^{-1}$ Na_2HPO_4, 11 $mmol \cdot L^{-1}$ glucose, 0.2% bovine serum albumin [Sigma-Aldrich, Deisenhofen, Germany], 5 $mmol \cdot L^{-1}$ *N*-(2-hydroxyethyl)piperazine-*N*′(2-ethanesulfonic acid), 50 $\mu g \cdot mL^{-1}$ penicillin, and 50 $U \cdot mL^{-1}$ streptomycin [Gibco BRL, Eggenstein, Germany]).

Sections were then incubated for 20 min with 250 μL of this buffer containing ^{125}I-labeled endothelin-1 (81,400 $GBq \cdot mmol^{-1}$; NEN-DuPont, Köln, Germany). For saturation binding assays, cross sections with 10, 20, 30, 40, 60, or 90 μm were incubated with ^{125}I-labeled endothelin-1 at various concentrations; for competitive binding assays, cross sections with 30 μm at 100 $pmol \cdot mL^{-1}$ (6 sections for each data point and animal) were incubated with ^{125}I-labeled endothelin-1 at various concentrations. Subsequently, sections were washed 3 times with ice-cold buffer and transferred to reaction cabs. Bound radioactivity was measured with a γ-counter (1282 CompuGamma CS; Pharmacia, Freiburg, Germany). Unspecific binding of endothelin-1 was determined in the presence of 1 $\mu mol \cdot L^{-1}$ unlabeled endothelin-1 (Sigma-Aldrich) and was subtracted from each data point.

For saturation binding assays, the binding of ^{125}I-labeled endothelin-1 to the surface of the cross sections at each concentration was determined through linear regression of bound endothelin-1 to section thickness. After the values of unspecific binding were subtracted, the values of specific binding were divided by the area of the measured cross sections. The maximum specific binding was calculated by nonlinear regression with the function $y = a \cdot x/(b + x)$ (SigmaPlot; SPSS Science Software GmbH, Erkrath, Germany, for Windows 5.0; Microsoft, Redmond, WA), where a equals the maximal endothelin binding revealing the density of overall (subtypes A and B) endothelin receptors. For competitive binding assays, the radioactive signals in the presence of unlabeled endothelins were calculated as the percentage of the signal in the absence of unlabeled endothelins.

Superfusion of Isolated Aortic Tissue Using Perfusion Chambers

The aortas were excised immediately after killing of the rabbits and were transferred to ice-cold buffer. The vessels were cut longitudinally between the branch points of the arteriae intercostalis and washed with ice-cold buffer. Plastic rings were fixed with Histoacryl (Braun, Melsungen, Germany) on the luminal side of the aortas. The segments of aortic tissue with the surrounding rings were cut and mounted in perfusion chambers that had been adapted from those developed by Ussing and Zehran (*26*). The first compartment of each chamber was connected through tubes to a buffer reservoir and a peristaltic pump. The second compartment was filled with buffer to prevent dehydration of the adventitia (Fig. 1). For equilibration, the aorta segments were first superfused for 20 min with buffer at room temperature, after which they were superfused for an additional 20 min with buffer either containing 100 $pmol \cdot L^{-1}$ ^{125}I-labeled endothelin-1 or 11 $nmol \cdot L^{-1}$ of the ^{99m}Tc-labeled endothelin derivative, also at room temperature. For the determination of unspecific binding of ^{125}I-labeled endothelin-1, 1 $\mu mol \cdot L^{-1}$ of unlabeled endothelin-1 was added. Subsequently, the segments were superfused for an additional 20 min with ice-cold buffer to wash the aortic segments. The aortic segments were

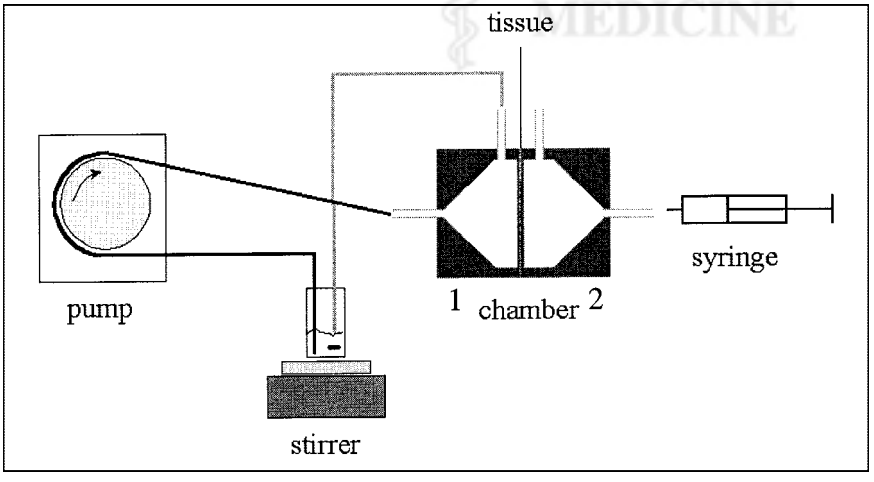

FIGURE 1. Perfusion apparatus with perfusion chambers adapted from those developed by Ussing and Zehran (*26*). In left compartment of perfusion chamber (compartment 1), aortic tissue is superfused with buffer containing ^{125}I-labeled endothelin-1 or ^{99m}Tc-labeled endothelin derivative through endothelium of vessel wall. Second compartment (compartment 2) is filled with buffer to prevent dehydration through adventitia.

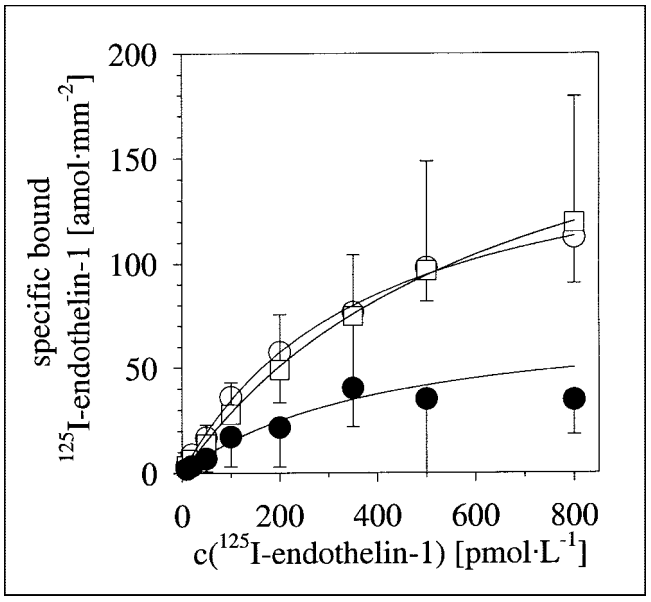

FIGURE 2. Specific binding of ^{125}I-labeled endothelin-1 to cross sections of rabbit aortas in saturation binding assays. ○ = normal aortas from NZW; □ = atherosclerotic aortas from balloon-denuded cholesterol-fed NZW; ● = atherosclerotic aortas from WHHL.

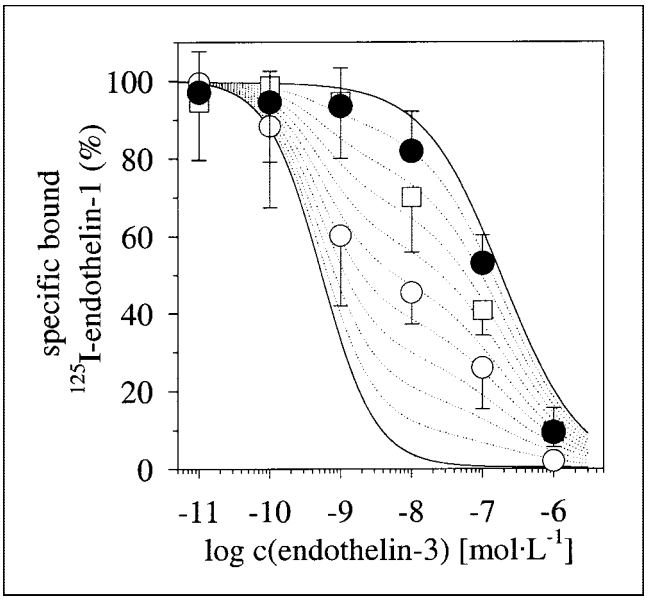

FIGURE 3. Competition of specific binding of ^{125}I-labeled endothelin-1 with unlabeled endothelin-3 on cross sections of rabbit aortas. ○ = normal aortas of NZW; □ = atherosclerotic aortas from balloon-denuded cholesterol-fed NZW; ● = atherosclerotic aortas from WHHL; solid lower line = competition of ^{125}I-endothelin-3 with unlabeled endothelin-3 on endothelin receptor subtype B as determined with cell line HH26CLN; solid upper line = competition of ^{125}I-labeled endothelin-1 with unlabeled endothelin-3 on endothelin receptor subtype A as determined with cell line A10; dotted lines = calculated competition curves on basis of solid black lines representing 10%–90% of each receptor type.

removed from the superfusion chambers, and the bound radioactivity was determined as described above.

RESULTS

Densities of Endothelin Receptors and Their Subtypes in Cross Sections of Rabbit Aortas

Cross sections of normal aortas derived from untreated and balloon-denuded aortas from cholesterol-fed NZW have similar densities—100 ± 4·10^6 receptors per square millimeter and 133 ± 5·10^6 receptors per square millimeter (subtypes A and B), respectively. On the contrary, the density of overall endothelin receptors is significantly decreased in cross sections of atherosclerotic WHHL (45 ± 12·10^6 receptors per square millimeter) as determined by nonlinear regression (Fig. 2; Table 1).

Unlabeled endothelin-3 competes for the binding of ^{125}I-labeled endothelin-1 to cell surfaces and to cross sections of rabbit aortic tissue. Endothelin-3 competes for the binding of endothelin-1 to the receptor subtype B at a concentration of approximately 10^{-11}–10^{-8} mol·L^{-1}, as determined with the rat endothelial cell line HH26CLN (Eurogentec, Seraing, Belgium) (Fig. 3). To compete for binding of endothelin-1 to subtype A, concentrations of higher than 10^{-8} mol·L^{-1} are needed (Fig. 3), as determined with the rat vascular smooth muscle cell line A10 (American Type Culture Collection, Rockville, MD). The calculated dotted lines, which represent 10%–90% of subtypes A and B, reveal that approximately 45% ± 10% of the endothelin receptors are of subtype A and 55% ± 10% are of subtype B in cross sections of untreated aortas from NZW. In cross

TABLE 1
Densities and Fractions of Endothelin Receptor Subtypes

	Receptors A + B	Subtype A		Subtype B	
Rabbit	$10^6 \cdot mm^{-2}$	$10^6 \cdot mm^{-2}$	%	$10^6 \cdot mm^{-2}$	%
NZW	100 ± 4	45 ± 11	45 ± 10	55 ± 11	55 ± 10
BD-NZW	133 ± 5	106 ± 16	80 ± 15	27 ± 16	20 ± 15
WHHL	45 ± 12	40 ± 13	90 ± 5	5 ± 13	10 ± 5

BD-NZW = balloon-denuded cholesterol-fed NZW.
Values were determined by saturation and competitive binding assays on cross-sections of normal and atherosclerotic aortic tissue.

sections of balloon-denuded aortas from cholesterol-fed NZW, the proportion of endothelin receptor subtype A increases to 80% ± 15%, whereas the proportion of subtype B decreases to 20% ± 15%. The proportion of subtype A in atherosclerotic aortas from WHHL also increased (90% ± 5%), whereas receptor subtype B is rarely detectable (5% ± 13%) (Fig. 3; Table 1).

Saturation and competitive binding assays show that, because of balloon denudation and the additional cholesterol diet, the expression of endothelin receptor subtype A is significantly upregulated (2.4-fold) compared with normal aortas of untreated rabbits. However, the density of receptor subtype A did not change in atherosclerotic aortas from WHHL, but the expression of endothelin receptor subtype B is dramatically downregulated in atherosclerotic aortas from WHHL (Table 1).

Accumulation of Endothelin-1 and the ^{99m}Tc-Labeled Endothelin Derivative in Superfused Aortas

Atherosclerotic aortas from WHHL and balloon-denuded cholesterol-fed rabbits accumulated 366 ± 132 amol·mm^{-2} ($P < 0.001$) and 338 ± 175 amol·mm^{-2} ($P < 0.002$), respectively, of the ^{99m}Tc-labeled endothelin derivative, nearly 3 times more than was accumulated by untreated normal aortas (137 ± 26 amol·mm^{-2}) (Fig. 4B).

^{125}I-labeled endothelin-1 binds to all 3 types of investigated aortas. Although the amount of unspecific binding is similar in all tested samples (15 ± 2 amol·mm^{-2} in normal and balloon-denuded NZW aortas and 14 ± 3 amol·mm^{-2} in WHHL aortas), specific binding is observed only in superfused normal (12 ± 9 amol·mm^{-2}) and balloon-denuded (19 ± 5 amol·mm^{-2}) aortas from NZW. Because the amounts of total and unspecific binding of endothelin-1 to atherosclerotic segments of WHHL are not significantly different (16 ± 2 amol·mm^{-2} and 14 ± 3 amol·mm^{-2}, respectively), no receptor-mediated endothelin-1 binding could be observed in these blood vessels (Fig. 4A).

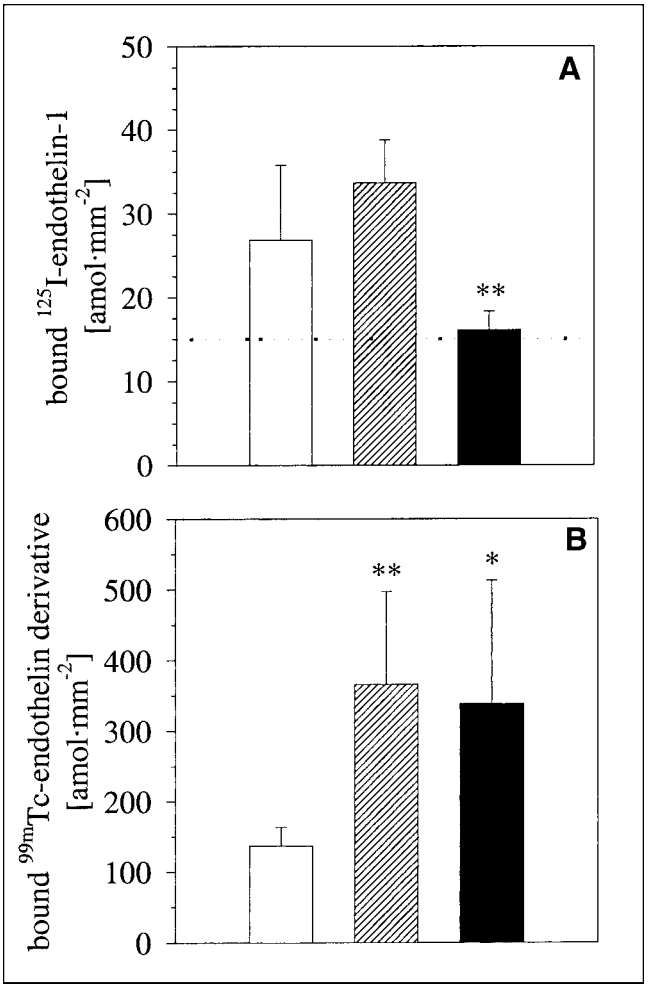

FIGURE 4. Binding of ^{125}I-labeled endothelin-1 (A) and ^{99m}Tc-labeled endothelin derivative (B) to superfused aortas. Dotted line in A represents amount of unspecific binding. Open bars = normal aortas from untreated NZW; hatched bars = atherosclerotic aortas from balloon-denuded cholesterol-fed NZW; solid bars = atherosclerotic aortas from WHHL. *$P < 0.002$; **$P < 0.001$.

DISCUSSION

Endothelins and their receptors seem to play an important role in atherosclerosis and restenosis. Besides their mitogenic effects on isolated cells (*15,27,28*), animal studies revealed antiatherosclerotic (*18,22*) and antirestenotic effects (*9,10,17,19*) of specific antagonists such as BMS-182874 and bosentan.

Previously, we reported that a ^{99m}Tc-labeled endothelin derivative could detect experimentally induced atherosclerotic lesions in balloon-denuded cholesterol-fed rabbits (*14*). Recently, we were able to show that the isolated complexes of ^{99m}Tc with this endothelin derivative (*29*) have moderate affinities to both endothelin receptor subtypes (*30*). In this study, we investigated whether any endothelin receptor subtype is overexpressed in atherosclerotic lesions and, therefore, may be useful for targeting and optimizing the specific binding of the endothelin derivative.

Two animal models for atherosclerosis were investigated: the cholesterol-fed NZW with balloon-denuded aortas and the WHHL. The first model is characterized by mechanical removal of the endothelium with a balloon catheter before being subjected to an additional cholesterol diet for 4–6 wk. The WHHL, which lacks receptors for low-density lipoprotein, serves as a model for familial hypercholesterolemia by spontaneously developing severe atherosclerosis after several months (*25*).

In both models, type A is the predominant endothelin receptor within the atherosclerotic vessels. The explanation for this distribution of endothelin receptor subtypes is different: In balloon-denuded aortas of cholesterol-fed NZW, subtype A increases, whereas in the atherosclerotic vessels of WHHL, expression of subtype B is suppressed. A comparison of the 2 models shows that it is more the ratio of the different endothelin receptor subtypes than the total amount

of endothelin receptors that is characteristic of their involvement in atherosclerosis. This observed predominance of endothelin receptor subtype A in atherosclerotic lesions is in line with the observed effectiveness of subtype A specific endothelin antagonists for the prevention of atherosclerosis and restenosis in animal studies (*9,10,18,19,22*).

For a scintigraphic approach to receptor imaging, the radiolabeled ligand needs to specifically accumulate in the diseased tissue. This is feasible in 2 ways. Either the target molecule is overexpressed in the diseased tissue, enabling enhanced specific binding of the diagnostic molecule to this area, or the diseased tissue has an increased permeability for the ligand. The latter could be caused by, for example, endothelial malfunction, which increases penetration of the ligand into the diseased area and leads to enhanced binding and, thus, disease-specific accumulation of the diagnostic molecule.

In the 2 investigated animal models for atherosclerosis, neither the density of overall endothelin receptors nor the density of either of the receptor subtypes is significantly increased in the atherosclerotic aortas. Thus, endothelin receptors do not support an enhanced accumulation of a ligand that is specific for endothelin receptors. Furthermore, the superfusion experiments have shown that the permeability of the normal blood vessel wall and its endothelium in untreated NZW is sufficient for binding of endothelin-1 to its receptors. Normal aortas of untreated rabbits and atherosclerotic aortas of balloon-denuded cholesterol-fed rabbits bind similar amounts of endothelin-1. Finally, as shown in the superfusion experiments, endothelin-1 does not bind in a receptor-mediated way to the atherosclerotic lesions of WHHL. This last result is probably not caused by decreased wall permeability but rather by plaque morphology, because the outer layers of the lesion contain high numbers of foam cells (*31*) and only a few smooth muscle cells, which express endothelin receptors. Therefore, increased permeability of atherosclerotic vessels could not be proven for endothelin-1 and probably also cannot be proven for its derivatives.

In spite of these data, the ^{99m}Tc-labeled endothelin derivative (*14,30*) binds specifically to atherosclerotic lesions of rabbits—not only to the previously described balloon-denuded aortas of NZW that received a normal or cholesterol-rich diet (*14,30*) but also to atherosclerotic aortas of WHHL as shown by superfusion experiments. The mechanism responsible for this clear discrimination between normal and diseased tissue remains unknown, but there is no doubt that the observed disease-specific accumulation of the technetium-labeled endothelin derivative is not mediated by binding to endothelin receptors either in superfusion experiments or in vivo.

CONCLUSION

Endothelin-1 is not able to discriminate between normal and atherosclerotic blood vessels, either because of overexpression of endothelin receptors or because of enhanced penetration of diseased tissue. We conclude that endothelin receptors are not suitable for molecular imaging of atherosclerosis. Although our previously described accumulation of a ^{99m}Tc-labeled endothelin derivative in atherosclerotic lesions could be shown again, this accumulation was most likely not mediated by endothelin receptor binding.

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