Evaluation of Indium-111-Polyclonal Immunoglobulin G to Quantitate Atherosclerosis in Watanabe Heritable Hyperlipidemic Rabbits with Scintigraphy: Effect of Age and Treatment with Antioxidants or Ethinylestradiol

Pierre N.M. Demacker, Tom P.J. Dormans*, Emile B. Koenders and Frans H.M. Corstens

Departments of General Internal Medicine and Nuclear Medicine, University Hospital Nijmegen, Nijmegen, The Netherlands

Scintigraphic detection of atherosclerotic lesions using ^{111}In-polyclonal IgG was studied. In Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model for hypercholesterolemia with spontaneous atherosclerosis, aged WHHL rabbits incorporated more ^{111}In-IgG into atherosclerotic lesions than young WHHL or control NZW rabbits. This result is in agreement with histological analysis. However, due to the low ratio of lesion-incorporated radioactivity to circulating radioactivity, in vivo gamma imaging of atherosclerosis with ^{111}In-IgG scintigraphy was unsuccessful. Interventional agents, Probucol or vitamin E, used for 28 days to reduce the amount of autoantibodies produced against biological modified low-density lipoproteins did not produce differences in ^{111}In-IgG incorporation into the aorta ex vivo. Ethinylestradiol, used for 28 days, exhibited similar incorporation with decreased serum cholesterol by 45%. Although atherosclerosis histology and lesion surfaces of WHHL rabbits are similar to those in adult humans, it is obvious that noninvasive gamma imaging with polyclonal ^{111}In scintigraphy is not reliable for serial evaluation of the extent of atherosclerosis. Our results emphasize the need to develop pharmaceuticals to image atherosclerosis.

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An early event in the development of atherosclerosis is the accumulation of lipid laden "foam cells" in the subendothelial space of the vessel wall. Recent studies have demonstrated that most of the foam cells are derived from monocyte-derived macrophages (*1–3*). Native low density lipoprotein (LDL) is not taken up by these macrophages, but biological modified LDL is internalized rapidly via scavenger receptors (*4,5*). Moreover, in vivo oxidation of LDL results in a grossly modified lipoprotein that rapidly accumulates within macrophages (*6*). Oxidation of LDL can be prevented by suitable antioxidants, which appear effective in reducing atherosclerosis progression in Watanabe heritable hyperlipidemic (WHHL) rabbits (*7*). Another intervention for preventing oxidative modification of LDL is treatment with ethinylestradiol (*8*), which also decreases serum lipids (*9*). Oxidized LDL is highly immunogenic. In WHHL rabbits and in cholesterol-fed New Zealand White (NZW) rabbits, increased concentrations of circulating autoantibodies were found (*10*). Intervention may decrease concentration of circulating LDL-antibody complexes and the amount of immune complexes incorporated into atherosclerotic lesions.

The clinical use of drugs designed to lower serum cholesterol and prevent LDL oxidation has increased the need for early lesion detection and therapy monitoring. Indium-111-labeled polyclonal immunoglobulin G (IgG) scintigraphy has been used successfully in the detection of early atherosclerosis induced in rabbits by endothelialization of the aorta with a balloon catheter (*11*) and in humans at sites of severe arterial narrowings (*12*). The rationale for this technique is based on the fact that macrophages trapped in the subendothelial space express large numbers of Fc receptors that can be imaged with molecules that bind to these receptors (*11*). On the other hand, circulating immune complexes can compete with this radiolabeled ligand. In this report, we describe efforts to optimize scintigraphic detection of atherosclerosis with ^{111}In-IgG. Noninvasive scintigraphic results were compared with ex vivo analyses of incorporated radioactivity as well as with histological staining.

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For reprints or correspondence contact: P.N.M. Demacker, PhD, Department of Medicine, Division of General Internal Medicine, University Hospital Nijmegen, P.O. Box 9101, The Netherlands.

*Present address: Department of Internal Medicine, Sint Joseph Hospital, Veldhoven, The Netherlands.

METHODS

Immunoglobulin Labeling

Human monospecific polyclonal IgG (Sandoglobulin, Sandoz AG, Nuernberg, Germany) was conjugated to diethylenetriamine-

pentaacetic bicyclic anhydride (bicyclic DTPA) according to the method described by Hnatowich et al. (*13*). Aliquots of the conjugate (0.5 ml) were radiolabeled with ^{111}In (indium chloride, Amersham International Ltd, Buckinghamshire, UK) via citrate transchelation. Radiochemical purity was determined by thin-layer chromatography (ITLC-SC Gelman Laboratories, Ann Arbor, MI) with 0.1 *M* citrate (pH 5.0) as the solvent. Labeling efficiency was always greater than 98%. The DTPA conjugate was diluted to 5 mg/ml with 0.15 *M* acetate (pH 6.5) and sterilized by membrane filtration (0.4 μm filter). Before injection, the syringes containing isotope solutions were counted. To accurately calculate the amount of radioactivity injected, the syringes were weighed both before and after tracer injection. The variation in injected radioactivity was usually less than 10%. In some cases, however, a deviation of more than 30% was observed, which could be corrected with the weighing procedure discussed in the Results section.

Animals

WHHL rabbits, bred by crossing and back crossing between WHHL males and New Zealand White (NZW) females (*14*), were used. The rabbits were fed rabbit laboratory chow containing 2% fat (LK04 diet, Hope Farms, Woerden, The Netherlands). The young WHHL rabbits used in the experiments were less than 6 mo of age; the adult WHHL rabbits were older than 18 mo. During the 28-day study period, adult rabbits were fed 1% of the antioxidant drug Probucol, 0.1% vitamin E or were treated with ethinylestradiol containing implants (*9*). The implants resulted in plasma ethinylestradiol concentrations that were 9 to 35 times above normal (*9*). Throughout the study period, body weights and the physical condition of the animals remained stable. The experiments were approved by the animal experimental committee of our institution.

Experimental Procedures

In Experiment 1, four young and four adult WHHL rabbits were studied and compared with four NZW rabbits. In Experiment 2, ^{111}In-IgG was injected into eight WHHL rabbits fed Probucol or vitamin E for 28 days and into eight untreated WHHL rabbits. In Experiment 3, the effect of ethinylestradiol treatment was studied in five WHHL rabbits in comparison with five WHHL controls and five untreated NZW rabbits.

Prior to imaging, each rabbit was tranquillized with 1 ml of Hypnorm. Blood samples were taken via venous ear punction. After the last imaging session, the animals were killed with a lethal dose of sodium pentobarbital. Blood and tissue samples were measured for ^{111}In activity in a LKB automatic well-type gamma counter. A sample of the originally injected preparation was used as a standard.

Imaging

Depending on the specific activity of ^{111}In-IgG, 25 μg protein, corresponding with 18–37 MBq, was injected into the left marginal ear vein of a standard rabbit weighing 2500 g. The doses were corrected for body weight. The mean weight of the animals was 2.8 kg (range 2.0–3.4 kg).

Scintigraphy was performed using a standard gamma camera with a parallel-hole, medium-energy collimator (Siemens, type Orbiter, Hoffman Estates, IL). Digital acquisition was recorded in a 256 × 256 matrix using a data processing system (Siemens Scintiview). Both the 173 keV and 247 keV ^{111}In gamma peaks with symmetric 20% windows were used. The animals were stretched on the surface of the collimator using a plastic frame to standardize their position during imaging. Anterior images were recorded at 2, 6, 24, 48 and eventually 72 hr postinjection for a preset time of 7.5 min (at 2 hr) and 10 min (at other time intervals). Typical images had 670–930 kcts at 2 hr and 410–540 kcts at 72 hr. For optimal imaging of the abdominal aorta, urine present in the bladder was removed by a catheter before imaging. The molecular weight distribution of fresh tracer and radioactivity present in serum 24 hr postinjection was analyzed by Sephadex G-25 chromatography using disposable columns (PD-10, Pharmacia-LKB).

Aorta Analysis

After the animals were killed, the heart and aorta were perfused with 300 ml of saline via a needle in the left ventricle and a cut in the right atrium. The entire aorta of each rabbit from the proximal carotid branches to the distal bifurcation was dissected and the adventitia were removed. Subsequently, the aortas were opened longitudinally and washed with saline. In Experiment 1, the aortas were washed five times by intensive mixing followed by an overnight soak in saline. In Experiments 2 and 3, the aortas were washed less intensively by mixing twice with saline for 5 min. After blotting on paper, the aortas were counted for radioactivity in a gamma counter.

The aortas were fixated with 10% formalin, stained with Sudan Red lipid stain (200 mg of stain dissolved in 100 ml of a 1/1 mixture of 70% ethanol and acetone) for 10 min and destained for 5 min in 50% ethanol (*15*). The aortas were then placed between glass plates and the lesion surfaces were estimated by visual inspection using the aortas of young and adult WHHL rabbits with known amounts of lesion surfaces as standards. The results obtained in the Experiments 1, 2 and 3 for the various groups of rabbits were averaged.

Statistical Analysis

Wilcoxon's rank sum test was used and values lower than 0.05 were considered significant.

RESULTS

Comparison of Serum Lipids and Lipoproteins

In comparison to NZW rabbits, serum cholesterol and triglyceride concentrations in young and adult WHHL rabbits were elevated 8-fold to 10-fold. This was due to an elevation of both the very low and intermediate density lipoprotein (VLDL + IDL, $d < 1.019$ g/ml) and of LDL concentrations ($1.019 < d < 1.063$ g/ml) (Table 1). Serum cholesterol decreased slightly in rabbits fed Probucol, but treatment with vitamin E did not affect serum lipids. In rabbits given ethinylestradiol, serum lipids decreased considerably, mainly in the VLDL + IDL-fraction.

Scintigraphic Imaging of Atherosclerotic Lesions

The conditions described in the Methods section were the result of preliminary experiments. For best results, the rabbits had to be fixed in a frame and their urine had to be catheterized from the bladder. Scintigrams of the anterior images of the abdomen of a NZW rabbit and a young and an adult WHHL rabbit 72 hr after intravenous injection of ^{111}In-IgG are shown in Figure 1. Differences in the amount of radioactivity present in the aorta in vivo could not be seen by three independent investigators in any of the experiments.

TABLE 1
Body Weight, Serum Lipids and Lipoprotein Concentrations

W	N	Body weight*	Cholesterol†	Triglycerides	VLDL + IDL-chol	LDL-chol
WHHL adults	17	2650	20.5 ± 2.1	3.8 ± 0.9	7.0 ± 3.1	9.0 ± 2.4
WHHL young	4	1980	16.0 ± 3.4	4.3 ± 2.3	N.D.	N.D.
WHHL vit. E	8	2513	18.9 ± 4.8	3.4 ± 2.2	6.7 ± 3.5	9.6 ± 3.6
WHHL Probucol	8	2530	14.7 ± 2.6‡	2.6 ± 0.7	6.6 ± 1.5	8.1 ± 1.8
WHHL ethinylestradiol	5	2840	7.4 ± 1.1§	1.0 ± 0.7§	1.6 ± 0.8§	10.3 ± 3.8
NZW	9	2780	2.1 ± 0.6	0.5 ± 0.1	0.2 ± 0.1	1.3 ± 0.5

*Average body weight in grams. The s.d. in all groups was less than 250 g.
†Concentrations (mean ± s.d.) in mmol/liter.
‡$p < 0.05$ vs. WHHL adults.
§$p < 0.01$ vs. WHHL adults.

Decay Rate and Homogeneity Analysis of Circulating ^{111}In-IgG

Figure 2 shows the decrease of whole blood radioactivity in control, new, young and aged WHHL rabbits and in those treated with Probucol or vitamin E. The half-life of ^{111}In-IgG in serum derived from a biexponentially decaying radioactivity time curve is approximately 5–6 hr, but a portion of the radioactivity is decayed at a slower rate. On Sephadex G-25 analysis, it appears that approximately 35% of the circulating radioactivity is recovered in fractions with a lower molecular weight than the original tracer at 24 hr postinjection (Fig. 3). Since release of the DTPA conjugate is not likely, these findings indicate degradation of IgG. The catabolized products were preferentially excreted in the urine (Fig. 3). No differences were observed in the distribution of radioactivity over both molecular weight fractions from WHHL and NZW rabbits 24 hr postinjection (data not shown).

In all experiments, the residual amount of radioactivity per gram of blood was similar 48 or 72 hr postinjection in all groups, except in the aged WHHL rabbits in Experiment 1 and in the NZW rabbits in Experiment 3 ($p < 0.01$). This proves that the correction procedure for the amount of tracer injected is adequate, at least when gross deviations from standard body weight are absent. Variations in residual blood radioactivity 48 or 72 hr postinjection within each group ranged between 4.5% and 16.6% (Table 2).

Incorporation of Radioactivity in the Aorta

The percent of the dose incorporated in the aorta ex vivo was extremely low in all groups, especially in Experiment 1. This is certainly due to the more vigorous washing procedure applied in this experiment. The between experiment differences implicate that only within experiment results can be compared. Accordingly, we observed in Experiment 1 that aged WHHL rabbits had incorporated twice as much radioactivity in their aortas ex vivo than young WHHL rabbits or NZW rabbits. Similarly, in Experiment 3, NZW rabbits had incorporated twice lower amounts of radioactivity in their aortas than WHHL controls. In Experiment 1, the difference in incorporated radioactivity remained when corrected for circulating amounts of radioactivity at 48 hr, but faded in Experiment 3 where initially decreased incorporated radioactivity was observed in NZW rabbits when compared to WHHL rab-

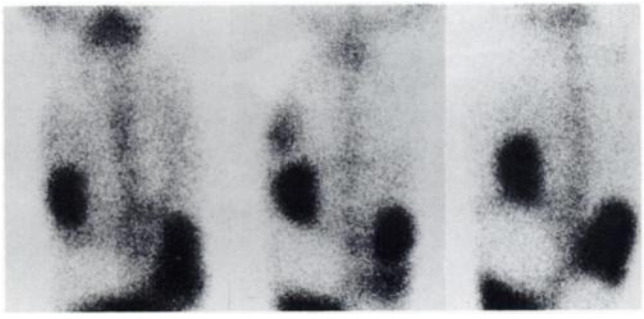

FIGURE 1. Scintigrams of anterior images of the abdomen of a NZW (left), a young WHHL (middle) and an aged WHHL (right) rabbit 48 hr after i.v. injection of ^{111}In-IgG. In all three images, spinal activity and residual blood-pool activity could not be distinguished. It is rather difficult to image aortic arch and thoracic lesions in both the anterior and lateral decubitus. Abdominal lesions do seem amenable to lateral imaging. Unfortunately, WHHL rabbits do not develop major lesions in descending aorta (*16*).

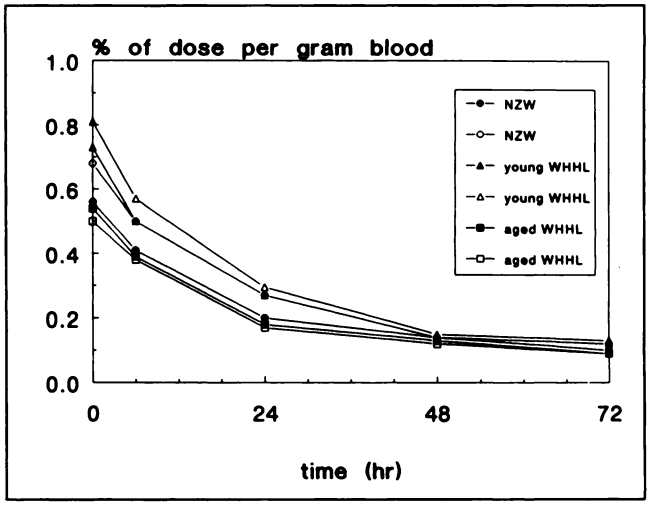

FIGURE 2. Radioactivity in the circulation after injection of ^{111}In-IgG in NZW rabbits and young and aged WHHL rabbits.

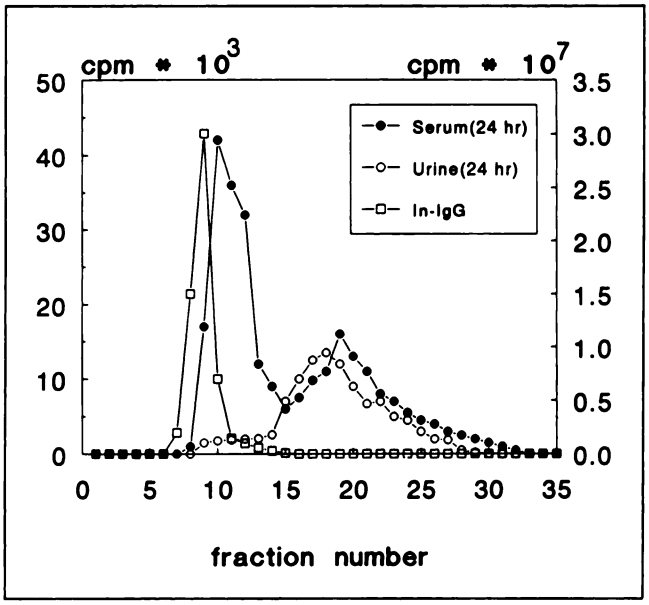

FIGURE 3. Stability study of ^{111}In-IgG. Serum and urine obtained 24 hr postinjection and freshly prepared tracer were subjected to Sephadex G-25 column chromatography and the fractions were counted for radioactivity. With these columns, the difference in the void volume is approximately 0.8 ml, which corresponds to one or two sampling tubes. The radioactivity present in the serum was distributed over two fractions: approximately 65% of the total radioactivity showed elution characteristics similar to the freshly prepared tracer, whereas approximately 35% of the radioactivity was eluted in fractions characteristic for lower molecular weight compounds. Note that elution of urine showed only one peak of radioactivity similar to the second peak present in serum. Left scale: serum and urine; right scale: ^{111}In–Ig6.

bits (Table 2). Note, that we had injected very reproducible amounts of radioactivity. Thus, correcting the incorporated radioactivity for residual circulating radioactivity seemed superfluous. It is clear that interventions with two different antioxidants or with ethinylestradiol do not reduce the amount of radioactivity incorporated in the aorta (Table 2).

When compared to histological identification of lesions (*15,16*), gamma images of incorporated radioactivity in the aortas of WHHL rabbits showed large interindividual variation. In addition, the incorporation was rather unevenly distributed. Generally, in line with histological staining (*16*), most of the radioactivity was present in the thoracal aorta with a gradual decrease in the direction of the bifurcation (Fig. 4). Histological detection of lesions was much more sensitive and specific. In agreement with earlier results (*15,16*), the lesion surfaces on the aortas of adult WHHL rabbits were between 8% and 10% compared to <1% in young WHHL rabbits and 0% in NZW rabbits. Within the experimental error of observation, the short-term interventions with both antioxidants or with ethinyl estradiol did not reduce lesion surfaces (Table 2). The data on lesion surfaces were related to the amounts of radioactivity in the aortas isolated from the various groups. Correction of the incorporated radioactivity for the residual circulating activity did not improve the agreement between radioactivity data and those obtained by histology (Table 2).

DISCUSSION

This study evaluates the possibilities of in vivo imaging of atherosclerosis. Thus, a reliable animal model for hypercholesterolemia and atherosclerosis, the WHHL rabbit, was used. This animal was studied at different ages and during interventions with relevant drugs known to decrease atherosclerosis. In addition to determining radioactivity incorporated in the aorta ex vivo, we expected to obtain insight into the feasibility of scintigraphic detection of atherosclerosis with ^{111}In-IgG.

Recent findings have shown that atherosclerosis has an immunological component with autoantibodies produced against biologically modified LDL detectable in the aorta

TABLE 2
Variation in the Percentage of Radioactivity per Gram Blood to the Total Dose Injected 48 to 72 Hours Postinjection

	n	Time (hr)	%Total dose/g blood	c.v.(%)	%Total dose/g aorta	Ratio incorporated/ circulated	Lesion surface
Experiment 1	4	48					
WHHL young	4		0.22 ± 0.01	4.5	0.008 ± 0.001	0.04	<1
WHHL aged	4		0.16 ± 0.01*	6.3	0.013 ± 0.002*	0.08*	10
NZW	4		0.20 ± 0.02	10.0	0.006 ± 0.001	0.03	0
Experiment 2		48					
Probucol	8		0.22 ± 0.02	9.1	0.03 ± 0.02	0.14	8
Vitamin E	8		0.24 ± 0.03	12.5	0.03 ± 0.01	0.12	8
WHHL control	8		0.23 ± 0.02	8.7	0.03 ± 0.01	0.13	8
Experiment 3							
WHHL ethinyl estradiol	5	72	0.22 ± 0.02	14.3	0.040 ± 0.004	0.33	8
WHHL control	5		0.23 ± 0.05	16.6	0.045 ± 0.005	0.33	8
NZW	5		0.21 ± 0.01†	5.8	0.026 ± 0.003†	0.29	0

*$p < 0.01$ vs. WHHL young or NZW rabbits.
†$p < 0.01$ vs. WHHL controls and WHHL ethinylestradiol.
Time = time at which the aorta was isolated.

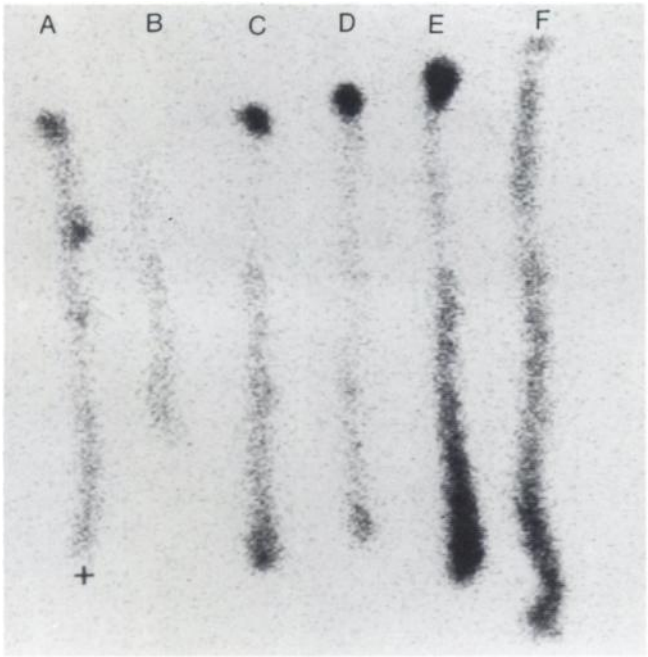

FIGURE 4. Ex vivo images of aortas of NZW rabbits, young and aged WHHL rabbits. The aortas of two NZW rabbits (A and B), of two young WHHL rabbits (C and D) and of two aged rabbits (E and F) were removed 48 hr after injection of the radiopharmaceutical. The arch is at the top and the bifurcation is at the bottom of the figure. The ex vivo images show a rather large within-group variability. Nevertheless, the aortas in rabbits E and F of Group 3 clearly show the greatest amounts of incorporated radioactivity.

and probably also in the circulation (*10,17,18*). The approach to detecting early atherosclerosis by tracing residual activated macrophages in the aorta (*11*) is innovative, but the autoantibodies produced against biological modified lipoproteins may compete with ^{111}In-IgG for binding to the Fc-receptor. This may hamper the interpretation of results obtained with a ligand for the Fc-receptor.

We were unable to image atherosclerosis with ^{111}In-IgG in the various groups of WHHL rabbits studied. This is in contrast to earlier observations in NZW rabbits after deendotheliazation of the aorta with a balloon catheter. In this model, early lesions could be imaged 48 hr after injection of ^{111}In-IgG. There are several reasons for this apparent discrepancy in the results in both studies. First, the scraped surface of deendothelialized NZW rabbits is probably more lesioned than the aortic segments in our WHHL rabbits, in whom lesion surfaces of a maximum 15% per aorta segment were reported (*15*). Consequently, the amount of incorporated radioactivity in our WHHL rabbits as determined ex vivo was extremely low: 0.013%–0.045% of the total injected dose per gram of aorta. This was at least seven times lower than the residual radioactivity in the blood. In view of the fact that the amount of incorporated radioactivity in the aorta ex vivo is of the same order as the standard deviation of residual blood radioactivity, the inability to image atherosclerotic lesions in WHHL rabbits with ^{111}In-IgG is clear.

In the radioactivity decay versus time curve, a slow, decaying component was present in the serum, which at 48 or 72 hr greatly contributed to the increased background radioactivity. It is likely that this is a physiological mechanism because the clearance of albumin (*19*), apoproteins (*20*) and lipoproteins (*21*) is also biexponential. If so, an improvement in the imaging of atherosclerosis can only then be achieved by increasing the incorporation of radioactivity in the target.

This incorporation of radioactivity in the aortas ex vivo in rabbits treated with antioxidants or ethinylestradiol was similar to that in control WHHL rabbits. It may be expected that these interventions may decrease the activation of subendothelially trapped macrophages and probably decrease the incorporation of ^{111}In-IgG in the aorta (*1,7–10*). However, the interventions used in this study also improve chemotaxis, which results in a decreased number of trapped macrophages and consequently decreased incorporation of radioactivity in the lesions. On the other hand, these interventions may decrease the production of autoantibodies against biologically modified LDL with consequently increased incorporation of ^{111}In-IgG due to decreased competition for Fc-receptor binding. The lack of any difference in the degree of lesion-incorporated radioactivity in treated and control WHHL rabbits is probably due to these counteracting mechanisms. These theoretical considerations emphasize the need for an in-depth study with relevant cell cultures in vitro as well as with turnover studies of antibody complexes. Obviously, measurement of circulating LDL antibody complexes is inadequate to obtain insight in any competition for Fc receptor binding in the subendothelial surface because these complexes are rapidly removed (at least partially) by similar Fc receptors on Kupffer cells in the liver.

The measurement of the degree of incorporated radioactivity in the aorta ex vivo as performed here has one important drawback; it is strongly dependent on the intensity of the washing procedure. Between experiments, the degree of incorporation of the label in the aortas of control rabbits varied twofold to sixfold. Apparently, trapped macrophages are partially removed by the washing procedure. On the other hand, if the aortas are washed less intensively, there is the risk of contamination with circulating and aspecific bound ^{111}In-IgG. This risk explains why our images of aortas ex vivo were inferior to the macroscopic staining pattern.

In summary, it was possible to demonstrate significant quantitative differences of radionuclide incorporation in young and aged spontaneous atherosclerotic lesions ex vivo with ^{111}In-IgG. Treatment of the rabbits with antioxidants and a hypolipidemic drug did not reduce accumulated radiotracer ex vivo. Although ^{111}In-IgG appears to be a valid tracer for detection of activated macrophages in tissues, it was impossible to detect early lesions in the aorta in vivo in WHHL rabbits because of a relatively low target-to-background ratio.

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EDITORIAL

Atherosclerosis Imaging: Pathophysiological Assessment for a New Era

In 1941, Arthur Master introduced the two-step electrocardiogram (*1*) and dramatically altered management decision-making in patients with chronic stable coronary artery disease. Previously, necessary prognostic inferences in such patients were based primarily on subjective complaints. With the new method, the choice of management options could be grounded on objective assessment of the functional consequences of coronary occlusions. In 1941, therapies were few and choices were limited. However, during the ensuing five decades, pharmacologic and surgical treatment modalities proliferated. Ultimately, some were shown to impact positively on the natural history of disease. In this context, easily applied, preferably noninvasive, periodically repeatable approaches to prognostication assumed ever greater importance.

Fortunately, as the need grew, many technical developments enhanced the capacity for noninvasive prognostication. Exercise electrocardiography continues to evolve in diagnostic and predictive accuracy, most recently as computer-based technology permits extraction of new nuggets of information from the sarcolemmal response to ischemia (*2*). However, the current standard for noninvasive prognostication involves radioisotopic imaging during stress to measure the functional importance of occlusive lesions. Beginning with brilliant pioneering efforts in perfusion scintigraphy (*3–5*), shortly thereafter supplemented by direct assessment of ventricular performance during exercise (*6*), scintigraphic stress testing has provided a sound objective basis

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For correspondence or reprints contact: Jeffrey S. Borer MD, New York Hospital-Cornell Medical Center, 525 East 68th St., F467, New York, NY 10021.

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