Radionuclide Imaging of Experimental Atherosclerosis with Nonspecific Polyclonal Immunoglobulin G

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The utility of nonspecific polyclonal IgG for external imaging of experimental atherosclerosis was tested in a series of rabbits after balloon catheter deendothelialization of the abdominal aorta. Following injection of ^{111}In-IgG, ^{111}In-Fc, or ^{111}In-Fab serial images were recorded. In addition, several animals received ^{125}I-low density lipoproteins [^{125}I-LDL], or ^{125}I human serum albumin [^{125}I-HSA] as positive and negative controls. Forty-eight hours after injection of the radiolabeled proteins, the aortas were removed, divided into abdominal and thoracic regions, counted, and autoradiographed. The images acquired after injection of ^{111}In-IgG and ^{111}In-Fc, showed clear focal accumulation of radioactivity in the healing abdominal aorta. In contrast, the images obtained after injection of ^{111}In-Fab did not show focal radionuclide accumulation. For ^{111}In-IgG and ^{111}In-Fc there were three to six times as many counts in the abdominal as in the thoracic aorta, while for ^{111}In-Fab and ^{125}I HSA, the abdominal and thoracic counts were nearly equal. The results suggest that radiolabeled IgG and Fc can be used to image experimental atherosclerosis.

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There is a major clinical need for a noninvasive test that could be performed serially to assess the presence and extent of atherosclerotic disease in patients. Such a test could be utilized to determine the efficacy of long-term therapy with cholesterol lowering drugs on the progression of disease, and screen symptomatic individuals prior to angiography, with an eye toward vascular surgery (*1,2*), thrombolytic therapy (*3*), or balloon angioplasty (*4*).

Early lesions of atherosclerosis encroach upon the vessel wall with minimal decrease in luminal diameter, and are thus difficult to detect angiographically. Moreover, it is at this early stage of development, when the lesions are in their most metabolically active state that medical intervention (i.e., lipid lowering drugs) or dietary manipulation may be most beneficial. Standard imaging techniques such as angiography, computed tomographic (CT) scanning, and magnetic resonance imaging are relatively ineffective for evaluating these early lesions. Similarly indium-111- (^{111}In) labeled platelets accumulate most effectively at regions of turbulent flow that are characteristic of advanced disease and are thus poor indicators of lesions that do not encroach substantially on the lumen (*5*). Imaging of the regional distribution of perfusion, such as with thallium-201 (^{201}TI) injected during exercise, requires lumenal narrowing of at least 50% to produce a regional deficit on the scan. To identify plaque early requires a different approach. Recently, radiolabeled (^{125}I or ^{99m}Tc) low density lipoproteins (LDL) have been employed as a means of detecting metabolically active plaques (*6–9*). While promising results were described in the early animal studies (*7*), experience with these radiopharmaceuticals in human subjects has not been encouraging (*8*).

Despite the fact that labeled LDL appears not to be the agent of choice for noninvasive imaging of early

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plaques, the concept of using a labeled compound that binds to some attribute of active atherosclerotic lesions remains appealing. In addition to lipoproteins, active plaques also contain foam cells. These foam cells are largely macrophage-derived and bear abundant Fc receptors that would be available to bind both nonspecific IgG and Fc fragments (*10*). Based on this information, we explored the use of radiolabeled IgG and Fc fragments prepared from the IgG in an attempt to image experimental atheroma (*11*).

In the present studies, we employed the healing balloon catheter deendothelialized rabbit aorta as an in vivo model of early atheroma. The results demonstrate that ^{111}In-labeled nonspecific polyclonal IgG and Fc fragments accumulate in the lesions at sufficient concentration for consistent external imaging.

METHODS

Preparation of Fc and Fab fragments

Human polyclonal IgG obtained from Sandoz Inc. (Sandoglobulin) (250 mg) was incubated with 2.5 mg of papain (Sigma) for 1.5 hr at 37°C. The reaction mixture was applied to a calibrated column of Sephadex G-100 and the fractions eluting in the 50 kDa range (Fc plus Fab) were pooled. The Fc and Fab fragments were then separated on a protein A Sepharose column. The fractions containing the Fc and Fab fragment were pooled, further purified by DEAE cellulose chromatography and stored at 4°C. The products were characterized by IEF, PAGE and Ouchterlong immunodiffusion.

Indium-111 Labeling

Intact IgG (Sandoglobulin) and Fc and Fab fragments were radiolabeled with ^{111}In via the DTPA-antibody chelate method (*12*). Briefly, isobutylchloroformate (0.25 mmol) was added to a cooled solution (ice bath) of the trietylamine salt of DTPA (0.25 mmol in 2 ml of acetonitrile) and the mixture stirred for 30 min. An aliquot of the resulting solution of the mixed carboxycarbonic anhydride of diethylenetriaminepentaacetic acid (DTPA) was then added to a cooled solution of intact antibody or fragment, dissolved in 0.1*M* $NaHCO_3$ with the pH maintained at between 7 and 8 by addition of 0.1*M* $NaHCO_3$. The mixture was then stored at 4°C overnight, dialyzed against 0.1*M* acetate buffer pH 5.0 and applied to a column of Sephadex G-25. The protein fraction was dialyzed against normal saline. A 0.5 ml aliquot of the resulting solution containing ~0.5 mg of protein was diluted with 0.5 ml of 1.0*M* citrate buffer (pH 5.0) and the solution combined with 1–2 mCi of $^{111}InCl_3$ (Amersham). The mixture was then incubated for 30 min at room temperature, applied to a column of Sephadex G-25 and the protein fraction isolated. The pooled protein fractions were passed through a 0.22-micron filter (Millipore Corp., Bedford, MA) prior to injection. The final specific activities of the ^{111}In-labeled proteins were between 2 and 4 μCi/μg. Chemical purity of the labeled products were determined by high performance liquid chromatography.

Protein Iodination

LDL (density = 1.025 to 1.050 g/ml) was prepared by sequential flotation in the ultracentrifuge (*13*). After dialysis against buffer containing 0.2*M* NaCl and 1.0 m*M* disodium ethylediaminetetraacetic acid, pH 8.6 (EDTA saline), the LDL was passed through a 0.22-micron filter. Lipoprotein purity was determined by double diffusion in agarose (*13*). Protein concentration was determined by the method of Lowry (*14*). Iodination of LDL was performed by a previously described modification of the McFarlane iodine monochloride technique (*15*). After dialysis against EDTA-saline, more than 97% of the radioactivity was precipitated by 10% trichloroacetic acid. The final specific activity was between 5.5 and 22.3 μCi/mg protein. Iodine-125-labeled HSA was prepared by the iodogen method (*16*).

Animals and Surgery

Male New Zealand white rabbits weighing 2 to 3 kg were obtained from ARI Breeding labs, West Bridgewater MA. They were maintained on a normal diet of Wayne rabbit ration (Allied Mills, Inc, Special Feed Division, Chicago IL). Their abdominal aortas were denuded of endothelium by a modification (*17*) of the Baumgartner technique (*18*). After each animal was anesthetized with ketamine and ether, the left femoral artery was isolated; a 4F Fogarty embolectomy catheter (Model 12-040-4F, Edwards Laboratories Inc., Santa Ana, CA) was introduced through an arteriotomy in the femoral artery and was advanced under fluoroscopic visualization to the level of the diaphragm. The catheter was inflated to a pressure of ~3 psi above the balloon inflation pressure with radiographic contrast media (Conray, Mallinkrodt, St. Louis, MO). Three passes were made through the abdominal aorta with the inflated catheter to remove the aortic endothelium before removal of the catheter, ligation of the femoral artery, and closure of the wound. The animals were returned to their cage, and allowed to heal.

Imaging Studies

Each radiolabeled protein (150–300 μCi of ^{125}I-LDL, 100 μCi of ^{125}I HSA or 500–1,000 μCi of ^{111}In-IgG, ^{111}In-Fc, or ^{111}In-Fab) was injected into the marginal ear vein of rabbits 4 to 5 wk after deendothelialization of the aorta. In all cases, the mass of injected antibody/fragment was ~250 μg. In some cases, serial blood samples were obtained from the opposite ear during the ensuing 48 hr and were analyzed for ^{125}I-LDL, ^{111}In-IgG, or ^{111}In-Fc.

Autoradiographs were prepared from the aortas of animals injected with both the indium and iodine labeled proteins, however, only those animals injected with the indium labeled proteins were imaged. At 6, 24, and 48 hr after injection of the indium labeled proteins, serial gamma camera images of the anterior abdomen were recorded with a standard field-of-view gamma camera (Ohio Nuclear, Series 100, Solon, OH) equipped with a medium-energy, parallel-hole collimator (set to record both photopeaks of ^{111}In). At the end of the last imaging session, each animal was injected with 4 ml of 0.5% solution of Evans blue dye (Allied Chemical Co., National Aniline Division, New York, NY) which stains areas of deendothelialized aorta blue (*19*). One hour later, the animals were killed by a lethal injection of sodium pentobarbital, the aorta was removed en bloc and washed free of blood with saline. The adventitia was removed completely and the remaining aorta was washed in formalin, divided into abdominal (ballooned) and thoracic (unballooned) regions, weighed and counted in an LKB automatic well-type gamma counter (LKB

model #1282). The aortic segments were then opened along the ventral surface pinned out, and photographed. The fixed, opened vessels were then covered with a single layer of plastic (Saran) wrap, placed on high speed x-ray film (Kodak Ortho-film OH-1), and stored for 2 to 3 wk in a Kodak "X-Omatic cassette" (24 × 30 cm) before development in a 90-sec X-OMAT processor.

Statistics

Statistical analyses (*20*) were performed by two-way analysis of variance followed by Duncan's new multiple range test.

RESULTS

Imaging

Gamma camera imaging of 25 rabbits that were injected with ^{111}In-IgG (n = 15), ^{111}In-Fc (n = 8), or ^{111}In-Fab (n = 2) was performed at 6, 24, and 48 hr after injection. The gamma camera images recorded at 6 hr revealed a typical blood-pool distribution with visualization of the heart, liver, spleen, kidneys, and great vessels. At 24 and 48 hr focal accumulation of the radiolabeled proteins was seen in the healing abdominal aorta. Figure 1 shows representative images of the anterior abdomen of ballooned rabbits 48 hr after injection of radiolabeled intact IgG (panel A) and radiolabeled Fc (panel B) demonstrating focal localization of radioactivity in the abdominal aorta. Panels C and D show similar scintigrams of a ballooned rabbit injected with radiolabeled Fab (control-1, panel C) and an unballooned rabbit (control-2) injected with intact IgG (panel D). In all the images, the general distribution of radioactivity is similar (cardiac blood pool, liver, spleen, kidneys, and bladder), however in the control animals, minimal accumulation of radioactivity is seen in the aorta.

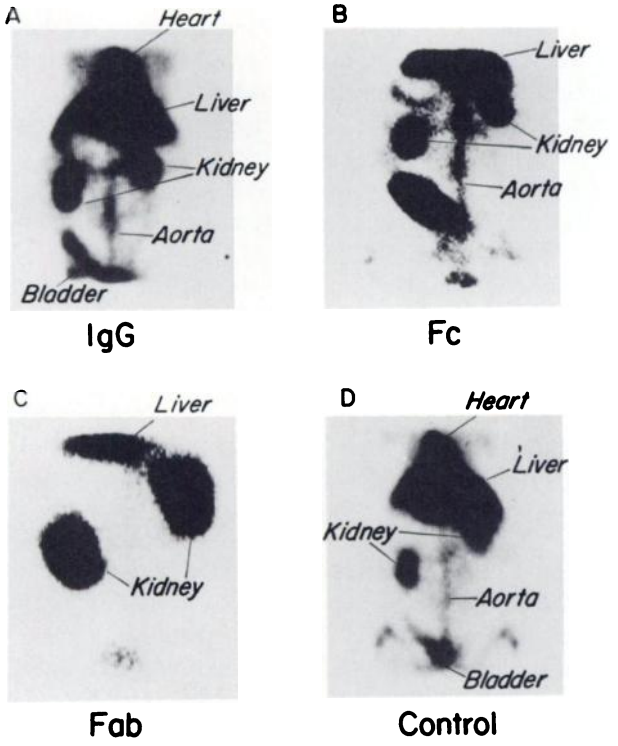

FIGURE 1
A–C: Scintigrams of the anterior abdomen of ballooned rabbits, 48 hr after i.v. injection of ^{111}In-IgG (A), ^{111}In-Fc (B) or ^{111}In-Fab (C). D. Scintigram of the anterior abdomen of an un-ballooned rabbit 48 hr after injection of ^{111}In-IgG. Similar results were obtained at 24 hr postinjection.

Tissue Counting

Measurement of radioactivity in the aortic segments of ballooned rabbits injected with radiolabeled intact IgG or Fc demonstrated three- to sixfold greater accumulation in the abdominal (ballooned) compared with the thoracic (unballooned) segment. This pattern was similar regardless of whether intact IgG or Fc was injected. However, the level of radionuclide accumulation in the healing abdominal aorta was greater for intact IgG than for Fc. Figure 2 shows the accumulation of ^{111}In-labeled intact IgG and Fc and ^{125}I-HSA in healing deendothelialized abdominal aorta and uninjured thoracic aorta, 48 hr after injection of the proteins. The effect of healing on radionuclide accumulation (% injected dose/gram, mean ± s.e.m.) was significant for intact IgG (0.0429 ± 0.003 for the intact thoracic aorta compared with 0.1700 ± 0.0172 for the healing abdominal aorta, $n = 11$, $p < 0.01$) and Fc (0.0345 ± 0.0028 for the intact thoracic aorta compared with 0.1026 ± 0.0073 for the healing abdominal aorta, $n = 8$ $p < 0.01$), but not for HSA (0.0369 ± 0.0030 for the intact thoracic aorta compared with 0.0481 ± 0.0079 for the healing abdominal aorta, $n = 6$, $p > 0.10$). Radionuclide accumulation in the healing abdominal aorta was 65.7% higher for ^{111}In-labeled intact IgG compared with Fc ($p < 0.01$). Radionuclide accumulation of the three proteins was not significantly different in the intact thoracic aorta. The radionuclide accumulation ratio (abdominal/thoracic) for IgG and Fc were not significantly different, but were significantly greater than the ratio for HSA ($p < 0.01$). The results obtained with ^{111}In-Fab were similar to ^{125}I-HSA (0.0398 ± 0.0082 for the intact thoracic aorta compared with 0.0285 ± 0.0038 for the healing abdominal aorta, $n = 2$). Due to the limited number of animals studied, the results obtained with ^{111}In-Fab could not be evaluated statistically. However, in another series of animals injected with monoclonal ^{111}In-Fab (data not shown), nearly identical results were obtained.

En Face Autoradiography

Evidence of focal sequestration of radioactivity in regions of regenerating endothelium came from the en face autoradiographs of the entire rabbit aorta (Fig. 3). The thoracic regions of the autoradiographic images from rabbits injected with either ^{125}I-LDL or ^{111}In-Fc were faint, diffuse, and relatively uniform in intensity.

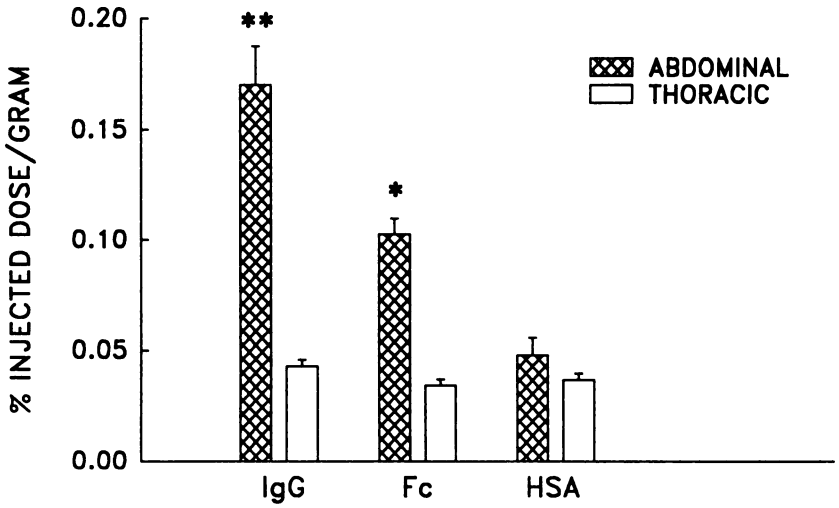

FIGURE 2
Accumulation of ^{111}In-IgG (n = 11), ^{111}In-Fc (n = 8), and ^{125}I-HSA (n = 6) (% injected dose/g, mean ± s.e.m.) in the aortas of rabbits, ~1 mo after deendothelialization of the abdominal aorta. The animals were killed 48 hr after injection of the radiolabeled proteins. The hatched bars represent healing abdominal aorta and the open bars, control thoracic aorta. * $p < 0.01$ compared to all other bars except the abdominal segment after IgG, ** $p < 0.01$ compared to the abdominal segment after Fc.

In reendothelializing abdominal aorta, three levels of radioactivity were apparent and each activity level, when compared to the Evans blue staining, corresponded to a specific anatomic region in the abdominal aorta. This pattern was the same for ^{125}I-LDL, ^{111}In-IgG, and ^{111}In-Fc. The centers of regenerating endothelial islands, which did not stain with Evans blue dye, had a very low level of radioactivity. The deendothelialized regions, which did stain with Evans blue, corresponded with areas of moderate radioactivity. The actively regenerating edges of the endothelial islands showed intense focal accumulation of radioactivity. Although there was some variation in the intensity of activity among different animals or different healing edges in the same vessels, there was no difference in the pattern of distribution of ^{125}I-LDL, ^{111}In-IgG, or ^{111}In-Fc in any of the aortic areas described.

In rabbits injected with ^{125}I-HSA or ^{111}In-Fab, the entire aorta showed an autoradiographic pattern similar to the thoracic pattern described above.

DISCUSSION

Experimental animal models have been used by many investigators to study the etiology of human atherosclerosis. With the healing, balloon-catheter deendothelialized rabbit aorta model, it has been demonstrated (*21,22*) that areas of most marked proliferative response with greatest lipid infiltration are confined to the leading edge of the regenerating endothelium, not to the deendothelialized areas of the aorta as had been expected. With the same model, other investigators (*23*) have found three times more free and esterified cholesterol in the reendothelialized vessel wall than in the pseudo-intima. In human vessels (*24*), a similar pattern has been found, with up to 12 times more LDL in human lesions covered with endothelium than in those that were not endothelialized. In previous studies, we showed that there was focal accumulation of radiolabeled LDL at the leading edge of the regenerating endothelium in healing ballooned rabbit arterial wall and that this accumulation is probably not mediated by high-affinity LDL receptors (*25*).

These studies indicate that the interaction of plasma LDL with regenerating endothelium is an important factor for lipid and lipoprotein accumulation in healing arterial wall. After initial interaction with arterial wall, one of the next steps in the generation of atherosclerotic plaques is the accumulation of lipid in foam cells. Since many of these cells are derived from macrophages, they would be expected to express Fc receptors on their surface. It has been shown (*10*) that foam cells isolated from the aortas of cholesterol fed rabbits selectively bind IgG, most probably by Fc-receptor interaction. In the present report, we present evidence that this interaction can be used to detect experimental atherosclerotic lesion by external imaging. Also, the pattern of radiolabeled intact antibody and Fc accumulation closely parallel the pattern of accumulation of labeled LDL.

While the ratio of antibody accumulation in abdominal (ballooned) to thoracic (control) aorta is only 3-6:1, this figure significantly underestimates the focal nature of the process. The focal nature of accumulation was apparent only in the autoradiographs (Fig. 3). For technical reasons, it was necessary to count the entire abdominal aorta. If one assumes that most of the radioactivity accumulation actually was confined to ~10% of the area counted, it is likely that the focal accumulation was far greater than three to six times the control.

The methods developed here might be applicable to imaging atherosclerotic lesions in human subjects. While the level of accumulation ^{111}In-IgG or ^{111}In-Fc are not markedly different than that for radiolabeled LDL preparations, the imperfect nature of animal models of atherosclerosis makes extrapolation to human atherosclerosis imaging difficult. Ultimately, only

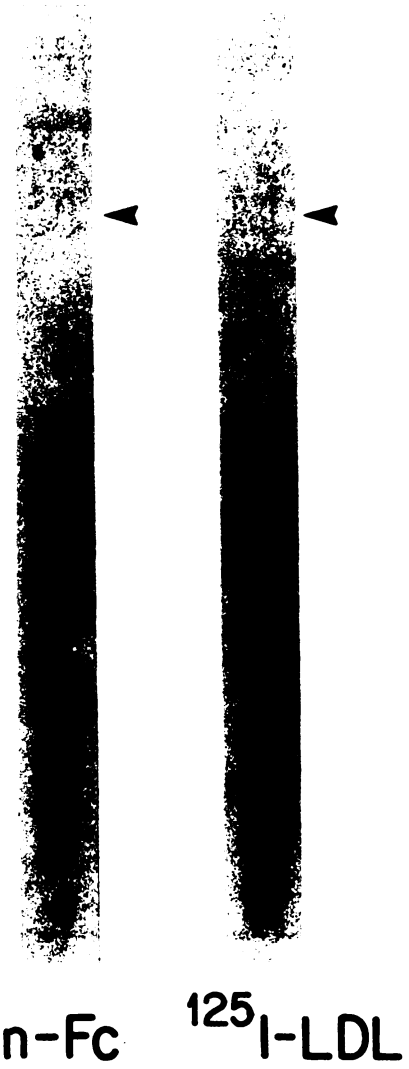

FIGURE 3
En face autoradiographs of the aortas from rabbits injected with ^{111}In-Fc (left) and ^{125}I-LDL (right). The labeled proteins were injected 1 mo after deendothelialization of the abdominal aorta and were allowed to circulate for 24 (right) or 48 (left) hr. One hour before removal of the aorta, Evans blue dye was injected. Comparison of the autoradiographs with the Evans blue staining showed that the areas of lowest radioactivity in the autoradiographs corresponded to the centers of endothelial islands in the abdominal aorta and to the entire thoracic aorta, which did not stain. The middle level of radioactivity corresponded with the areas that lacked endothelial coverage and thus did stain. The most pronounced areas of radioactivity occurred at the leading edge of the endothelial islands where active regeneration was in progress. The arrows indicate the upper border of the thoracic aorta.

human imaging experiments can determine which agent is superior. However, because of the ready availability and ease of preparation of the antibody based reagents, they are clearly superior from a practical point of view. The polyclonal human IgG preparation utilized here is already licensed for therapeutic purposes in the treatment of immunologic disturbances. The safety of this preparation at doses many times higher than those needed for imaging is well documented. Thus, evaluation of ^{111}In-IgG in humans could proceed quite quickly. Ultimately, labeled Fc fragments or polymers of Fc may prove to be better imaging agents because of their shorter half-life in the circulation and hence a potentially higher target/background ratio.

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