In Vivo Hypertensive Arterial Wall Uptake of Radiolabeled Liposomes

Howard N. Hodis, John K. Amartey, Donald W. Crawford, Emily Wickham, and David H. Blankenhorn

Using five sham-operated and seven aortic coarctation-induced hypertensive New Zealand White rabbits intravenously injected with neutral small unilamellar vesicles loaded with \(^{[111]In}\)nitrilotriacetic acid, we demonstrated in vivo that the normal aortic arterial wall participates in liposome uptake and that this uptake is increased in the hypertensive aortic wall by approximately threefold (\(p \leq 0.0001\)). Among the three regions examined, aortic arch, thoracic aorta, and lower abdominal aorta, the difference in uptake between the normotensive and hypertensive arterial walls was significantly different, \(p \leq 0.05\), \(p \leq 0.0001\), and \(p \leq 0.05\), respectively. The uptake by the different regions of the hypertensive arterial wall is consistent with the pathological changes present in these areas. Furthermore, the extent of liposome uptake by the aortic wall is strongly correlated with the height of the blood pressure (\(r = 0.85\), \(p = 0.001\), \(n = 11\)). We conclude that neutral small unilamellar liposomes can be used to carry agents into the arterial wall in vivo in the study of hypertensive vascular disease and could be especially useful for the delivery of pharmacologically or biologically active agents that would otherwise be inactivated within the circulation or are impermeable to the arterial wall. (Hypertension 1990;15:600–605)

A method for delivering biologically and pharmacologically active substances that are either impermeable to the arterial wall or inactivated within the circulation could be potentially useful as a tool in the study of certain metabolic processes of hypertension at the arterial wall cellular level in vivo.\(^1\)\(^2\)

Liposomes have the greatest potential in fulfilling such a task as they can entrap, protect, and deliver many types of pharmacologically and biologically active substrates capable of altering intracellular processes both in vitro and in vivo.\(^3\)\(^4\)

The tissue distribution of small unilamellar vesicles after intravenous injection has been studied extensively. Liposomes are cleared from the circulation rapidly by the monocyte/macrophage cells of the mononuclear phagocyte system (reticuloendothelial system) located in the liver, spleen, lung, bone marrow, and monocytes within the circulation.\(^5\)\(^6\) In addition, liposomes have been identified by uptake studies to be located in the lymphatic system, kidney, heart, intestine, stomach, fat, brain, and skeletal muscle.\(^7\) Because the high uptake by most of these tissues overshadows that of the arterial wall, the importance, and thus usefulness, of liposome uptake by the arterial wall has not been explored.

Although much effort has been put forth to determine if liposomes enter the extravascular space by passing out of the circulation through capillaries,\(^6\)\(^7\) studies examining the actual uptake of liposomes by the vessel wall in vivo are sparse.\(^8\)

The present study examined the question of whether, and to what extent, a large vessel, namely the aorta, takes up liposomes. Herein, we describe the in vivo uptake of radioactively labeled liposomes by the normal aortic arterial wall and the pronounced increase in this uptake by the hypertensive arterial wall.

Because of this uptake we propose that liposomes could be used as a tool to carry pharmacologically and biologically active agents into the arterial wall in vivo to study hypertensive vascular disease at the cellular level; they could be especially useful for the delivery of macromolecules that need protection from inactivation within the circulation or are impermeable to the arterial wall.
Methods

Animal Preparation

Seven male New Zealand White rabbits (3.00±0.24 kg) were given general anesthesia (2 ml ketamine [100 mg/ml]-xylazine [20 mg/ml] i.m. every hour), and, using sterile operative technique, hypertension was induced by partial ligation of the portion of the aorta between the superior mesenteric artery and diaphragm with a silk suture. Partial ligation was determined when a thrill distal to the suture was palpable.

Five other male New Zealand White rabbits (2.80±0.37 kg), representing the sham-operated group, underwent the same surgical procedure as the hypertensive group except that the aortic suture was loosely placed around the aorta and not tightened.

All procedures and handling of the rabbits and tissue were the same in the hypertensive and sham-operated groups. All the rabbits were fed a regular rabbit-chow diet.

Blood Pressure Measurements

Indirect systolic blood pressure measurements were obtained by the ear capsule translumination method, which has been shown to be linearly correlated with the mean intra-arterial pressure. These measurements were obtained in a darkened room by the same observer after the rabbits were calm and warmed. The measurements were obtained preoperatively and each week postoperatively for the duration of the experiment including the day the rabbits were killed. Each blood pressure value is the result of an average of at least five successive measurements.

Liposome Preparation

The liposomes and the kit to load the liposomes with indium-111 were a gift from Vestar, Inc., San Dimas, California. The 111In (in 0.05 mol/l HCl) was purchased from DuPont (Boston, Massachusetts). The liposomes used in this study were small unilamellar vesicles (<0.1 μm) specially prepared with the divalent ionophore A23187 incorporated into the lipid bilayer and the chelator nitrilotriacetic acid (NTA) entrapped within the aqueous core. These liposomes were prepared from distearoyl phosphatidylcholine and cholesterol in a 2:1 molar ratio by sonication.

Loading of 111In into the preformed liposomes was performed by incubating the mixture at 80° C (±2° C) for 30 minutes (±3 minutes). Vesicle loading was terminated by the addition of 0.1 ml of 10 mM sodium ethylenediaminetetraacetic acid (EDTA). Paper chromatography performed on each mixture, with Whatman 3 mm paper and 10 mM EDTA in 0.9% sodium chloride as the developing solution, revealed an 85%–95% loading efficiency, consistent with the results of Mauk and Gamble.

Uptake Experiments

Thirty-five days postoperatively, each rabbit was injected intravenously with a bolus of 30 mg indium-111-labeled liposomes/kg body wt containing a total of 500–600 μCi 111In radioactivity. Four days after the indium-111-labeled liposomes were injected, the rabbits were killed by a pentobarbital overdose. The complete aorta from the valve ring to the common iliac-caudal artery trifurcation was then dissected from the rabbits. The adventitia was sharply dissected away and the aortic tissue repeatedly washed in physiological saline to remove all the blood. The aortic tissue was then dried and weighed on an analytical balance. Radioactivity counting was done in a gamma-well counter set at a window of 150–300 keV (111In photopeaks: 173 keV and 247 keV) for 2 minutes. The radioactivity within the standards (obtained at the time of injection) and the aortic tissue was determined simultaneously for each rabbit.

The whole aorta without the suture (removed en bloc by cutting 1 mm above and below the suture) was counted and then divided into three sections: aortic arch, thoracic aorta, and lower abdominal aorta. Each section was weighed and counted separately. The defined sections were from the valve ring to the distal end of the brachial artery (aortic arch), from the end of the aortic arch to the 1 mm point above where the suture was removed (thoracic aorta), and from the 1 mm point below where the suture was removed to the common iliac-caudal artery trifurcation (lower abdominal aorta).

All uptake values were standardized for the percent injected dose of 111In per gram of aortic tissue to ensure accuracy in all comparisons.

Histologic Procedures

Microscopic examination of the aortic tissue was performed on all the rabbits. Tissue samples were prepared by perfusion fixation with glutaraldehyde at 80 mm Hg for 30 minutes. Histology sections were cut 6 μm thick and stained with hematoxylin and eosin. The aortic tissue from the hypertensive and sham-operated rabbits was processed together. Photomicrographs were taken with a Zeiss photomicroscope.

Statistical Analyses

The absolute and percent change in blood pressure from the preoperative baseline to the fifth postoperative week was calculated for each rabbit. Differences from zero in mean percent change were tested within groups with one-sample t tests. Differences in mean percent change were tested across groups with an independent t test. Blood pressures obtained at the fifth postoperative week were correlated with the whole aorta indium-111-labeled liposome uptake by using a Pearson product-moment correlation. Differences between groups in indium-111-labeled liposome uptake and tissue weight for each aortic location were tested using independent t tests. A location difference in indium-111-labeled liposome uptake
Table 1. Blood Pressure Changes From Baseline to Week Five

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 0</th>
<th>Week 5</th>
<th>Change</th>
<th>% Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT (n=6)</td>
<td>69.9±5.0</td>
<td>81.5±2.1</td>
<td>11.7±4.3</td>
<td>20.0±9.0*</td>
</tr>
<tr>
<td>SO (n=5)</td>
<td>72.0±2.4</td>
<td>64.1±4.1</td>
<td>-7.9±3.3</td>
<td>-11.0±4.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM. HT, hypertensive group; SO, sham-operated group.

*p<0.05, hypertensive group vs. sham-operated group; within group percent change.

between the aortic arch, thoracic aorta, and lower abdominal aorta in the hypertensive group was tested with a one-factor analysis of variance. One extremely high value in the hypertensive group was tested for being an outlier by the Grubbs procedure, and deleted from the analysis. All analyses were performed by using the Statistical Analyses System (SAS) on a mainframe IBM system.

Results

Table 1 summarizes the blood pressure changes for the two experimental groups. The hypertensive group (n=6) experienced a significant percent increase in blood pressure (20.0%, p=0.02) from the preoperative baseline to the fifth postoperative week. The sham-operated group (n=5) experienced a nonsignificant percent decrease (-11.7%, p=0.06) in blood pressure over the same 5-week time period. The percent change in blood pressure between the two experimental groups is significantly different (p=0.02).

Uptake of indium-111-labeled liposomes by the whole aorta is significantly greater (p=0.0001) in the hypertensive group than in the sham-operated group (Table 2). Furthermore, at each location analyzed (aortic arch, thoracic aorta, and lower abdominal aorta), uptake in the hypertensive group was significantly greater than the sham-operated group (Table 2).

Although examination of the data for the hypertensive aorta suggests an ascending trend in indium-111-labeled liposome uptake from lower abdominal aorta to thoracic aorta to aortic arch (Table 2), the locations were not significantly different (p=0.20).

No overlap in uptake of the radioactively labeled liposomes occurred between any of the hypertensive and sham-operated rabbits (Figure 1). This lack of overlap reaffirms the strong statistical difference in indium-111-labeled liposome uptake between the two experimental groups. Between the two experimental groups, there is a minimum twofold to a maximum fourfold difference in uptake (Figure 1).

Finally, there is a strong statistically significant correlation (r=0.85, p=0.001, n=11) between the blood pressure measured at the fifth week and indium-111-labeled liposome uptake by the whole aorta (Figure 2).
FIGURE 3. Histomicrographs of representative cross sections of aortic arch (panel A), thoracic aorta (panel B), and lower abdominal aorta (panel C), from a hypertensive rabbit. Arch and thoracic regions come from above site of coarctation, lower abdominal region from below. Note normal architecture of arterial wall in lower abdominal aorta (panel C) with no intimal thickening and increased cellularity. Intimal changes in both thickness and cellularity increases from thoracic aorta (panel B) to aortic arch (panel A). Many round cells with the appearance of macrophages are present within intima of both these regions. Compared with normal one cell-thick intima of respective arterial wall regions taken from same sample sites from sham-operated animal (Figure 4A and 4B), these lesions become more obvious. Perfusion fixed and stained with hematoxylin-eosin; bar=50 μm.

Compared with the sham-operated rabbits, when the aortas from the hypertensive rabbits were dissected from the animals they were observed to be stiffer and weigh slightly more (0.85±0.07 vs. 0.73±0.06 g). Compared with the sham-operated rabbits, the intima of the arterial wall from the hypertensive rabbits was markedly thickened and composed of an increased cellularity including cells with the appearance of macrophages (Figures 3A and 3B and 4A and 4B). It is noteworthy that the aortic portion of the hypertensive arterial wall below the coarctation (lower abdominal aorta) is morphologically indistinguishable from the sham-operated aortic regions, all composed of a normal intimal thickness and normal appearing endothelium (Figures 3C and 4A, 4B, and 4C). Also of note is that the degree of intimal thickening and cellular involvement of the hypertensive aortic wall increases from lower abdominal aorta to thoracic aorta to aortic arch (Figure 3A, 3B, and 3C).

Discussion

The results of this study show that intravenously injected liposomes are taken up by the normal aortic wall in vivo and that this uptake is significantly increased twofold to fourfold when hypertensive arterial wall changes are induced. Liposomal uptake appeared to be related to the extent of hypertensive arterial wall changes. Uptake of the indium-111-labeled liposomes increased in the same direction as the hypertensive arterial wall thickening and intimal cellular involvement, increasing proximally from the lower abdominal aorta to the aortic arch (Figure 3A, 3B, and 3C). Furthermore, arterial wall uptake of the liposomes was strongly correlated with the height of blood pressure. Data from other laboratories has shown that uptake of radiolabeled albumin by the canine aortic wall in hypertension occurs by a similar gradient and that a similar correlation exists between blood pressure changes and carbon particle deposition in rat mesenteric arteries.

Although endothelial denudation secondary to partial ligation of the aorta leading to liposome uptake is a possibility, several pieces of evidence weigh heavily against this as an explanation for the uptake of liposomes in the hypertensive arterial wall. First, liposome uptake was found far from the coarctation (i.e., in the aortic arch). Furthermore, if endothelial denudation did occur, it would be expected to have been most extensive at the site of ligation and liposome uptake greatest in this area. In fact, however, liposome uptake increased in a direction rostral (away) from the ligation (Table 2). Second, the comparison of liposome uptake between the aorta with and without the area containing the ligation (the area expected to have the greatest denudation and fibrotic changes) showed no statistical difference. In the hypertensive arterial wall, this uptake was 12.5±4.4×10^-3% vs. 11±4.9×10^-3% of total admin-
Because of the absence of leakage of $^{111}\text{In}]NTA$ from liposomes and release of free unencapsulated $^{111}\text{In}$ back into the circulation after tissue uptake, $^{111}\text{In}]NTA$ is a reliable marker used for studying total intact liposome uptake by tissues.\textsuperscript{7,8,13,16-21} Other investigators have shown by gamma ray-perturbed angular correlation studies that the type of $^{111}\text{In}]NTA$-labeled liposomes used in this study maintain their structural integrity and that $^{111}\text{In}]NTA$ does not leak from the liposomes while in the circulation.\textsuperscript{7,8,22,23} When $^{111}\text{In}$ is released from liposomes within tissue and cells, it avidly binds to protein and remains fixed at the site of liposome uptake.\textsuperscript{13,18,20,21,24} Furthermore, kinetic studies of the uptake of $^{111}\text{In}]NTA$-labeled liposomes indicate that no free $^{111}\text{In}$ is released into the circulation after tissue degradation as the release of free $^{111}\text{In}$ from tissue to blood is not detectable.\textsuperscript{8,18-21}

Because the uptake values were standardized for tissue weight, this had the potential of confounding the final results, especially because the sham-operated aortic tissue weighed less than the hypertensive aortic tissue. The difference in tissue weight between the two experimental groups is consistent with the pathological changes that occur in hypertension and, to our knowledge, has not been commented on before. However, even when normalized to tissue weight, the difference in indium-111-labeled liposome uptake between the hypertensive and sham-operated groups remained highly significant.

The mechanisms by which liposomes gain entrance into the arterial wall is unclear. However, it has been shown that hypertension enhances arterial wall permeability to serum components and macromolecules.\textsuperscript{17,25} Whether liposomes are small enough to use these permeability changes is unknown. Alternatively, it is known that liposomes are taken up by monocytes within the circulation\textsuperscript{6} and that monocytes attach to the endothelium and then migrate into the subendothelium of the hypertensive arterial wall.\textsuperscript{17,25,27} The dual involvement of the monocyte cell in liposome uptake and hypertensive arterial wall lesion formation could account for the arterial wall accumulation of liposomes. Furthermore, other investigators have shown that liposomes are taken up by aortic endothelium in vitro and have suggested that this occurs either by adsorption or endocytosis.\textsuperscript{2}

Whatever the explanation for the increased uptake of liposomes by the hypertensive arterial wall, it probably is not solely related to the elevation of blood pressure. This is demonstrated by the fact that liposome uptake below the coarctation was significantly different between the hypertensive and sham-operated groups (Table 2). A similar phenomenon has been observed by Huttner et al\textsuperscript{25} who showed that, above the site of aortic ligation in rats, passage of protein tracers is dependent on the level of blood pressure elevation. Below the coarctation, however,
protein tracers could be found within the endothelium at a normal blood pressure and within the subendothelium at a subnormal blood pressure indicating a phenomenon not directly related to blood pressure elevation. We assume that the blood pressure in the hypertensive rabbits below the coarctation was lower than that above, which the histology appears to confirm (compare Figure 3A and 3B with 3C and Figure 3C with 4A, 4B, and 4C). Therefore, a lower than normal blood pressure below the coarctation in the hypertensive group as compared with the sham-operated group is a plausible explanation for the difference in liposome uptake in this region of the aorta. However, the possibility of submicroscopic or molecular changes occurring in the normal-appearing arterial wall below the coarctation in the hypertensive animals induced by hormonal or local factors leading to a greater liposome uptake cannot be excluded.

These points need clarification and the actual mechanism of arterial wall liposome uptake in vivo needs further exploration. We do believe, however, that liposome uptake occurs from the lumen as rabbit aortic intima and media contain no demonstrable vasa vasorum.

With the large body of data showing that liposomes can be used as a macromolecule-carrying system, we believe that liposomes can be used as a vehicle to protect and carry agents into the arterial wall, especially the hypertensive arterial wall in which liposome uptake is markedly increased.

Acknowledgments

We thank Dr. Richard Proffitt for supplying the liposomes and for his technical advice, Stephanie Bevan for her assistance with the preparation of the animals, and Rosie Baca for the preparation of this manuscript.

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Key Words • coarctation hypertension • macromolecule carrier systems • liposomes • rabbit studies
In vivo hypertensive arterial wall uptake of radiolabeled liposomes.
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Hypertension. 1990;15:600-605
doi: 10.1161/01.HYP.15.6.600

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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