Liposome accumulation in ischaemic intestine following experimental mesenteric occlusion

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Partial intestinal ischaemia was produced by ligation of selected primary laterals of the mesenteric artery in the rat. Both positively and negatively charged liposomes (multiply labelled with $^{99m}$Tc]diethylenetriamine pentaacetic acid, [$^3$H]methoxy-inulin, and [$^{14}$C]-cholesterol), administered 24 h following ligation, were accumulated in ischaemic (necrotic) intestine.

Liposomes (concentric phospholipid bilayer vesicles) have been widely studied as potential carriers of therapeutic agents in a variety of clinical disorders (1,2). In this context, Caride & Zaret (3) studied the myocardial distribution of multilamellar liposomes containing $^{99m}$Tc]diethylenetriamine pentaacetic acid (DTPA) 24 h following experimental coronary occlusion in dogs. $^{99m}$Tc-Label administered entrapped in neutral or positively charged liposomes was shown to accumulate in ischaemic myocardium; there was an inverse linear correlation between $^{99m}$Tc-label distribution and regional myocardial blood flow. Accumulation was dependent on the net charge of the liposomal membrane: $^{99m}$Tc]DTPA encapsulated in negatively charged liposomes was distributed passively as a function of regional blood flow. The purpose of this present study was to establish whether liposomal accumulation is unique to ischaemic myocardium or is a general feature of ischaemia per se irrespective of anatomical site. The experimental model selected was the irreversibly ischaemic rat intestine.

Methods

Insertion of an inlying left ventricular catheter

Microspheres, liposomes, and unentrapped radiolabels were injected through an inlying catheter inserted via the right carotid artery into the left ventricle. The catheter was constructed of PE-10 tubing fused by hot air to PE-90 tubing. Blood pressure was recorded by connecting the catheter, during insertion, to a Bentley pressure transducer coupled to an oscilloscope. The change from an arterial to an intraventricular blood pressure signal allowed the tip of the

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catheter to be located and secured in the left ventricle. The extracorporeal element of the catheter was passed underneath the skin so as to emerge through the intrascapular region and was exteriorized through a stainless steel helicoid spring (4). The free end of the spring was passed through a restraining hole in the top of the cage so as to allow unrestricted movement of the animal within the confines of the cage. The rats used were male Fischer or Sprague-Dawley rats (250–300 g; fed ad libitum).

Production of intestinal ischaemia

A section of the small intestine was exposed through a right abdominal incision. Primary laterals of the mesenteric artery supplying a 10- to 12-cm length of intestine were double-ligatured and cut between the points of ligation. The intestine was placed back in the abdominal cavity and the incision sutured. Catheterization and mesenteric ligation were performed under ether anaesthesia.

Determination of regional intestinal blood flow

Regional intestinal blood flow prior to mesenteric ligation was determined by the injection of \(^{51}\)Cr-labelled microspheres; and that post-ligation (approximately 10 min prior to death), by the injection of \(^{85}\)Sr-labelled microspheres. Microspheres in 10% dextran in saline (0.9% w/v) (number injected \(\geq 3 \times 10^5\); diam. 15 \(\pm\) 3 \(\mu\)m; 3M Company) were injected via the catheter: intraventricular administration of microspheres was obviously essential to guarantee first-passage clearance of microspheres by peripheral (including mesenteric) capillary beds. The post-mortem distributions of \(^{51}\)Cr- and \(^{85}\)Sr-labels in intestinal samples provides a measure of regional intestinal blood flow pre- and post-ligation. No attempt was made to calculate absolute blood flows (ml/min\(^{-1}\)). Results are expressed as % of the injected dose adjusted to per g tissue (\(\pm\) S.E.M.).

Preparation of liposomes

Liposomes (33 \(\mu\)mol lipid in 2 ml chloroform per preparation) were prepared with [4-\(\text{C}\)cholesterol (New England Nuclear, 54 mCi/mmol\(^{-1}\); 2.5 \(\mu\)Ci) with lipid compositions as specified below. After rotary evaporation of the lipid mixtures in chloroform, the dry lipid films were suspended in 2 ml of 0.9% saline containing [\(\text{H}\)]-methoxy-inulin (New England Nuclear, 432 mCi\(\cdot\)g\(^{-1}\); 100 \(\mu\)Ci) and [\(\text{Tc}\)DTPA (prepared with Medi-Physics Inc. DTPA kit using 1–5 mCi [\(\text{Tc}\)vertexetate]. Liposomes were sonicated for 6 min at room temperature (Heat Systems-Ultrasonics bath sonicator). Liposomally entrapped material was separated from free material on a 25- x 1.5-cm Sepharose 2B column immediately prior to administration to animals. The lipid concentration was adjusted to 5 \(\mu\)mol/ml\(^{-1}\), and 0.5 ml (2.5 \(\mu\)mol lipid) was injected into each animal. The encapsulation of \(\text{H}\)- and \(\text{Tc}\)-labels was approximately 0.7 and 1.8% respectively. Positively charged liposomes were composed of lecithin:stearylamine:cholesterol, 8:1:1; negatively charged liposomes, of lecithin:dicetylphosphate:cholesterol, 7:2:1 (molar ratios).
Determination of radioactivity

The radioactivity present in tissue samples was counted as follows: gamma emitters ($^{99m}$Tc, $^{51}$Cr, and $^{85}$Sr) were counted in a Beckman Gamma 8000 gamma-counter. Tissue samples were oxidized (Biological Material Oxidizer purchased from R.J. Harvey Instrument Corp., Hillsdale, N.J.) and the resultant $^{14}$CO$_2$ (derived from [4-$^{14}$C]-cholesterol) and $^{3}$H$_2$O (derived from $[^3]$Hmethoxy-inulin) counted in a Searle Analytic 92 scintillation counter. Statistical analysis was by the Student t-test. Results were calculated as % of the injected dose adjusted to per g tissue (+ S.E.M.).

Results and Discussion

To study the interaction of liposomes with ischaemic intestine, a model was adopted involving ligation of selected primary laterals of the mesenteric artery in the rat. Regional intestinal blood flows were determined before and after mesenteric occlusion using radiolabelled microspheres ($^{51}$Cr- and $^{85}$Sr-labelled respectively). Approximately 24 h after mesenteric ligation, animals received one of four possible treatments. (1) To 7 rats, positively charged liposomes were injected via the inlying catheter, whilst (2) a second group of 6 rats received negatively charged liposomes. Liposomes were prepared with [4-$^{14}$C]cholesterol and contained $[^3]$Hmethoxy-inulin and $[^9m]$Tc-DTPA encapsulated within the aqueous compartment (see Methods), (3) A third group of 10 rats received $[^3]$Hmethoxy-inulin (100 µCi) and $[^9m]$TcDTPA (approximately 2.5 mCi) in 0.9% saline (0.5 ml), i.e. not entrapped in liposomes (hereafter referred to as free). (4) To ascertain whether the presence of liposomal lipid per se alters the in vivo distribution of free marker compounds, a fourth group of 5 rats received $[^3]$Hmethoxy-inulin and $[^9m]$TcDTPA mixed with (but not entrapped within) positively charged liposomes.

Rats were sacrificed 2 h following liposome/marker administration by a lethal dose of sodium pentobarbital. On necropsy, a blackened necrotic section of intestine could be clearly delineated. Adjacent to this, but still clearly within the region that would otherwise have been perfused by the ligated mesenteric laterals, were sections of intestine with marked inflammation. These were identified, for convenience, as 'peri-necrotic'. Necrotic, peri-necrotic, and normal intestine were cut into small sections, cleaned, and their radioactivity counted.

It is clear from Table 1 that the experimental protocol provided a reproducible model of intestinal ischaemia. Irrespective of the experimental group, mesenteric ligation resulted in marked ischaemia in the intestine designated necrotic. There were significant differences in the pre-occlusion ($^{51}$Cr-label) regional blood flows between the experimental groups. Nevertheless, the residual flows ($^{85}$Sr-label) in the necrotic regions of the four groups were similar. Mesenteric ligation resulted in reactive hyperaemia in the normal and peri-necrotic intestinal regions. Indeed, there was no statistically significant difference between the apparent blood flows of normal and peri-necrotic regions. The reactive hyperaemia was a specific response to intestinal ischaemia per se; sham-operated animals (results not shown) showed no statistically significant increase in regional blood
Table 1. Regional intestinal blood flow following mesenteric occlusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Microspheres</th>
<th>Intestinal region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Peri-necrotic</td>
</tr>
<tr>
<td>1. Positives</td>
<td>7</td>
<td>$^{51}$Cr</td>
<td>2.75±0.42 (28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{85}$Sr</td>
<td>4.59±0.42</td>
</tr>
<tr>
<td>2. Negatives</td>
<td>6</td>
<td>$^{51}$Cr</td>
<td>1.33±0.12 (24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{85}$Sr</td>
<td>4.07±0.42</td>
</tr>
<tr>
<td>3. Free</td>
<td>10</td>
<td>$^{51}$Cr</td>
<td>1.53±0.22 (38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$^{85}$Sr</td>
<td>3.51±0.29</td>
</tr>
<tr>
<td>4. Free Plus</td>
<td>5</td>
<td>$^{51}$Cr</td>
<td>1.47±0.14 (16)</td>
</tr>
<tr>
<td>positive liposomes</td>
<td></td>
<td>$^{85}$Sr</td>
<td>3.51±0.38</td>
</tr>
</tbody>
</table>

The values for regional blood flow are expressed as % of the relevant injected microsphere dose adjusted to per g tissue (± S.E.M.). The numbers in brackets refer to the number of samples. The distributions of $^{51}$Cr- and $^{85}$Sr-microspheres are direct measures of regional blood flow pre- and post- ligation respectively.
flow post-operatively ($^{81}$Cr- and $^{85}$Sr-label distribution was more or less identical).

Having shown the reproducibility of the experimental model, we can now turn our attention to the central question of liposomal uptake by normal and ischaemic intestine. Three radioactive marker compounds were used: one associated with the liposomal membrane ([4-$^{14}$C]cholesterol), and two ([99mTc]DTPA and [$^{3}$H]methoxy-inulin) encapsulated within the aqueous compartment of the liposome. Use of any one single marker has proven limitations. Cholesterol incorporated into liposomal lipid is known to exchange with cholesterol in cell plasma membranes in vivo (2), whereas DTPA is of a sufficiently low molecular weight that it diffuses continuously out of the liposome. The use of the multiple labelling technique described permits one to deduce with some measure of confidence the in vivo fate of liposomes from the distribution of $^{14}$C-, $^{3}$H-, and 99mTc-labels.

The data obtained relating to the distribution of liposomal 99mTc-, $^{3}$H-, and 14C-labels in normal and ischaemic intestine are shown in Table 2. To simplify the presentation of data, the uptake of labels by peri-necrotic intestine is not shown: statistical analysis showed that peri-necrotic and normal intestine were more or less identical in their uptake of radiolabels. Injection of free [99mTc]DTPA and [3H]methoxy-inulin (i.e. not encapsulated in liposomes) resulted in minimal uptake ($<$0.2% injected dose $\cdot$g$^{-1}$ tissue) by normal intestine. There was an approximately two-fold ($P <0.01$) greater $^{3}$H- and methoxy-inulin uptake by necrotic intestine. The inference is that ischaemia/necrosis, as would be expected, results in increased membrane permeability.

Encapsulation of [99mTc]DTPA and [3H]methoxy-inulin in positively or negatively charged liposomes substantially increased their intestinal uptake. This applied irrespective of regional intestinal blood flow. However, uptake of the two labels did not increase in parallel in that there was a consistently greater uptake of 99mTc than there was of $^{3}$H. The increases in label uptake by intestine as a result of their encapsulation in liposomes was statistically highly significant and there can be no doubt that liposomal entrapment increases intestinal uptake of entrapped aqueous phase markers. This increased uptake of [99mTc]DTPA and [3H]methoxy-inulin is dependent on their entrapment in liposomes. Free [99mTc]DTPA and [3H]methoxy-inulin injected simultaneously with positively charged liposomes (labelled with [4-$^{14}$C]cholesterol) were taken up by normal and ischaemic intestine in a manner analogous to free [99mTc]DTPA and [3H]methoxy-inulin injected alone (results not shown).

It is clear (Table 2) that intestinal uptake of liposomes (or, more strictly speaking, radiolabels associated with or entrapped within liposomes) was increased in ischaemia/necrosis. The apparent uptake of 99mTc-label by necrotic intestine was greater than three-fold that by normal (or peri-necrotic) intestine. This difference ($P <0.0001$) applied equally whether the [99mTc]DTPA was entrapped in positively or negatively charged liposomes. The increased uptake of 99mTc-label by ischaemic intestine was paralleled by analogous increases in $^{3}$H and $^{14}$C uptake. The uptake of $^{14}$C- and $^{3}$H-labels from positively charged liposomes by necrotic intestine was approximately twice that by normal intestine ($P$ values $<0.0001$ and 0.02 respectively). Similar
Table 2. Uptake of $[^{99mTc}]$DTPA and $[^{3}H]$methoxy-inulin, administered either free or entrapped in positively or negatively charged liposomes (labelled with $[^{4-14C}]$cholesterol), by intestine following mesenteric occlusion

<table>
<thead>
<tr>
<th>Label</th>
<th>Intestinal region</th>
<th>Free</th>
<th>Positive liposomes</th>
<th>Negative liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{99mTc}]$DTPA</td>
<td>Normal</td>
<td>0.096±0.01 (36)</td>
<td>0.61±0.06 (24)</td>
<td>0.40±0.04 (24)</td>
</tr>
<tr>
<td></td>
<td>Necrotic</td>
<td>0.22±0.03 (25)</td>
<td>1.82±0.28 (14)</td>
<td>1.21±0.18 (19)</td>
</tr>
<tr>
<td>$[^{3}H]$Methoxy-</td>
<td>Normal</td>
<td>0.106±0.01 (32)</td>
<td>0.25±0.04 (24)</td>
<td>0.28±0.04 (24)</td>
</tr>
<tr>
<td>inulin</td>
<td>Necrotic</td>
<td>0.157±0.02 (24)</td>
<td>0.55±0.13 (14)</td>
<td>0.40±0.08 (18)</td>
</tr>
<tr>
<td>$[^{4-14C}]$</td>
<td>Normal</td>
<td>-</td>
<td>0.71±0.06 (24)</td>
<td>0.65±0.11 (24)</td>
</tr>
<tr>
<td>Cholesterol.</td>
<td>Necrotic</td>
<td>-</td>
<td>1.33±0.12 (14)</td>
<td>1.02±0.30 (19)</td>
</tr>
</tbody>
</table>

Results are expressed as % of the injected dose of radionuclide·g tissue$^{-1}$ (± S.E.M.). The numbers in brackets refer to the number of samples. The italic figures are the $P$ values for the differences between the treatments indicated by the lines. NS means not statistically significant ($>0.05$).

Differences in $^{14}C$ and $^{3}H$ uptake between necrotic and normal intestine resulted when these labels were associated with negatively charged liposomes, but statistical analysis of these preliminary data showed the differences not to be significant ($P>0.05$).

In the ischaemic myocardium, Caride and Zaret (3) showed selectivity in liposome uptake to be dependent on the net charge borne by the liposome, i.e. $[^{99mTc}]$DPTA encapsulated in positively charged and neutral liposomes was accumulated, whereas that encapsulated in negatively charged liposomes was distributed passively as a function of regional blood flow. There does not appear to be an analogous clear-cut difference between the interactions of positively and negatively charged liposomes with ischaemic intestine. Liposomal entrapment, irrespective of the net charge of the liposome membrane,
resulted in an increased uptake by ischaemic and, to a lesser extent, normal intestine. Whatever the difference(s) therefore between the interactions of positively and negatively charged liposomes with intestine, it is not an absolute difference(s) but only a question of degree. Uptake of radiolabels associated with positively charged liposomes was greater than the corresponding uptake from negatively charged liposomes, and the differences are statistically significant (P <0.05).

Given that liposomal entrapment increases the uptake of encapsulated or membrane-incorporated radiolabels by ischaemic intestine, liposomes obviously warrant attention as potential carriers of therapeutic or diagnostic agents in vascular disorders of the intestine. It is conceivable that the efficacy vis a vis ischaemic intestine of antibiotics, radionuclides for diagnostic scintigraphy, membrane-stabilizing agents (corticosteroids, chlorpromazine), etc. might be increased by liposomal entrapment. Injected liposomes in vivo are predominantly phagocytosed and metabolized by the reticuloendothelial system. This uptake severely limits the number of liposomes reaching other sites in the body and has emerged as the major practical drawback relating to the use of liposomes in various forms of chemotherapy (2). In quantitative terms, we were able to show that liposomal entrapment resulted in substantial delivery of entrapped or membrane-incorporated compounds to ischaemic intestine, i.e. up to 1.82% of injected dose g intestine⁻¹. Consequently, the fact that the reticuloendothelial system will take up the bulk of any injected liposomes does not necessarily detract from the feasibility of using liposomal delivery systems in intestinal ischaemia.

The mechanism(s) of liposome uptake by ischaemic and normal intestine has not been elucidated. Indeed the mechanism need not be the same for the two tissue types. That the 99mTc-, 14C-, and 3H-labels were not taken up in a constant l:h:l ratio tends to argue against endocytosis of intact liposomes as the primary mechanism. Electron microscopic studies of ischaemic myocardium given positively charged liposomes 2~ h following coronary occlusion (5) are in accord with this conclusion. In their interaction with tissues, liposomes undergo variable degrees of endocytosis, fusion with the plasma membrane (6,7), absorption, and molecular exchange, no single mechanism being exclusive. Prolonged ischaemia inevitably results in cellular necrosis with the resultant release of metabolites and other cell components. The interaction of liposomes with released phospholipases or other cell components (e.g. lysophosphatides (8) and Ca2+ ions) may result in destruction or destabilization of the liposomal membrane. This lysis/destabilization would be localized at the site of tissue necrosis. Consequently, liposomes may be viewed as behaving as microprobes sensitive to the biochemical environment and responding to changes in this environment by specific and non-specific structural alterations. These alterations presumably result in leakage of encapsulated markers, dissolution of the lipid bilayer, membrane fusion with cell membrane, etc. Small molecules in the aqueous compartment like [99mTc]DTPA may become free and interact with Ca2+ ions or other structures and thereby accumulate in areas of necrosis in a manner analogous to [99mTc]pyrophosphate (9). Inulin molecules likewise will diffuse into the extracellular compartment and, due to
the partial disruption of the cell membrane, diffuse into the cytosol of necrotic cells. The differences in the distributions of [3H]methoxy inulin and [99mTc]DTPA may well be related to differences in molecular size, rates of diffusion, and/or their interactions with Ca2+ ions, proteins, and other cell components. It is viewed as unlikely that any single cell type is exclusively responsible for liposome accumulation in necrosis, although it cannot be entirely precluded that cellular infiltrates (macrophages, PMN leucocytes, lymphocytes, etc.) play a major role.

Acknowledgements

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References