Transendothelial Transport of Low Density Lipoprotein in Association with Cell Mitosis in Rat Aorta

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Atherosclerosis is characterized by focal areas of lipid accumulation and intimal smooth muscle cell proliferation in large arteries. In vivo studies on rat aorta with Evans blue-albumin conjugate (EBA) have shown that there are preferential sites of increased permeability with an increased uptake of the conjugate. It has been shown that these blue areas are associated with a high endothelial cell turnover rate and an enhanced permeability to lipids. In a previous study, we demonstrated that 99% of endothelial cells in the mitotic (M) phase as identified by hematoxylin staining of the dividing nuclei exhibited EBA leakage and that these dividing cells accounted for 30% of all leakage sites. In the present study, experiments were performed on the thoracic aortas of 10 adult male Sprague-Dawley rats to determine the statistical frequency of isolated leaks to Lucifer yellow-low density lipoprotein conjugate (LY-LDL) at the level of individual cells and to assess the relationship of such leaks to the cell turnover processes. Leakage of LY-LDL around individual endothelial cells was visualized by fluorescence microscopy, and cells in mitosis on the same specimens were identified by hematoxylin staining. Although endothelial cell mitosis is infrequent (0.034%), 80% of dividing cells in the M phase were associated with LY-LDL leakage. These dividing cells accounted for 45% of all leakage spots. These findings lend support to our recent hypothesis that transiently open junctions surrounding the endothelial cells undergoing cell turnover provide pathways through which LDL enters the subendothelial space, resulting in lipid accumulation. (Arteriosclerosis 9:230–238, March/April 1989)

Atherosclerosis is characterized by the deposition of lipids, the formation of foam cells, and the proliferation of smooth muscle cells in the arterial wall. Animal experiments have shown that there are certain areas in the aorta that are preferentially stained by Evans blue-albumin conjugate (EBA) after its intravascular administration. These blue areas have been shown to be associated with a high endothelial cell turnover rate and an enhanced permeability to lipids. These local areas also correspond to regions of low shear stress with flow reversal and are sites prone to the development of atherosclerosis. The subendothelial accumulation of unesterified cholesterol has been suggested as the very initial event in atherogenesis. However, the mechanisms and pathways by which low density lipoprotein (LDL) enters the arterial wall remain enigmatic.

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The endothelial injury hypothesis suggests that endothelial injury is responsible for the initiation of the atherosclerotic disease processes. This classical theory of atherogenesis has been critically re-evaluated in light of extensive morphological studies with electron microscopy, vascular casting methods, or time-lapse video microscopy. The absence of overt endothelial denudation in either endoxin-treated or lipid-fed animals during the early stage of lesion formation, and the fact that the dying endothelial cells are gradually sloughed off from below by movement of neighboring healthy endothelial cells have led to the postulation of a new hypothesis for the localization of atherosclerosis, which links endothelial cell turnover, leaky junctions, enhanced macromolecular permeability, and LDL metabolism. This hypothesis suggests that the endothelial cells involved in cell turnover have open or poorly organized junctions whose functional protein strands are either disrupted or have not yet fully formed. These transiently leaky junctions provide the pathway for the passage of large macromolecules (e.g., LDL) into the arterial wall. This theory can explain the local enhancement of lipid accumulation in regions of aorta where the hemodynamic pattern is complex, endothelial cell turnover rate is high, and atherosclerosis is prone to develop.

In a recent short-term, in vivo study on the rat aorta in which EBA was used as the macromolecular tracer, and hematoxylin staining was used to identify endothelial cells in mitosis, we were able to show the close correlation between EBA leakage and endothelial cell mitosis at the level of individual cells. Almost all (99%) endothelial cells that could be morphologically identified as being in the
mitotic (M) phase were associated with EBA leakage, and these mitotic cells accounted for 30% of all cellular leakage sites, even though the mitotic cells constituted only 0.014% of the total cell population. The transient opening of a junction around the mitotic cell is assumed: 1) to occur gradually from a normal spacing to a gap that is sufficiently wide to allow the passage of macromolecules the size of LDL or larger, 2) to be maintained with this wide gap for a certain period, and 3) then to gradually return to normal size. Therefore, it would be interesting to study how the partitioning of the total number of leakage sites between the mitotic and nonmitotic cells changes with tracer molecular size. In the present study, we used LDL as a macromolecular tracer to test the influence of molecular size on the correlation between junctional leakage and cell mitosis.

Methods

Preparation of Lucifer Yellow-Low-Density Lipoprotein Conjugate Solution

Fluorescence-labeled LDL was prepared with the use of Lucifer yellow VS (LYVS, obtained from Sigma Chemical Company, St. Louis, MO). Conjugation was carried out by gently mixing 5 mg of LYYS in 2 ml of 0.1 M Na2CO3 with 10 mg of human LDL in 2 ml of 0.15 M NaCl (protein concentration 5 mg/ml; obtained from Sigma Chemical Company) at room temperature for 2 hours. The mixture was then dialyzed (12K to 14K cutoff value) against phosphate-buffered saline at pH 7.4 in the cold room (40°F) overnight to remove unconjugated LY dye. The final volume of LY-LDL conjugate solution was approximately 4 ml with a protein concentration of 2.5 mg/ml.

Animal Experiments

Ten adult male Sprague-Dawley rats weighing approximately 300 g were used in this study. The experiments were performed under pentobarbital anesthesia (30 mg/kg i.p.). The right femoral artery and the left femoral vein were cannulated with 22 G needle catheters. A total of 4 ml LY-LDL solution was slowly injected into the left femoral vein. Approximately 9 minutes later, 1000 IU of heparin was injected intravenously, the chest was opened quickly, and the heart was exposed. At 10 minutes after LY-LDL injection, the rat was sacrificed with an overdose of pentobarbital injected intravenously; at the same time, a 22 G needle catheter was inserted into the left ventricle via a cardiac puncture and was connected to a pressure reservoir set at a physiological pressure of 100 mm Hg. The catheter placed in the right femoral artery was used as an aegis route for perfusion. A buffered saline solution was perfused first via the left ventricular needle catheter at 100 mm Hg pressure until the emergence of clear fluid from the aegis site (approximately 10 seconds). The perfusate was then switched to 10% formaldehyde, which was perfused for 20 minutes for preliminary fixation. After perfusion fixation, the aorta was excised between the aortic root and the diaphragm. The cuneiform-shaped specimen was immersed in 10% formaldehyde overnight. The thoracic aorta was then excised and divided into pieces. Each aortic piece was cut open longitudinally and pinned onto a dental wax plate with the endothelial surface facing up. The specimens were stained with Harris' hematoxylin for 30 seconds. The adventitial tissue was carefully removed, and the specimen was mounted wet onto a glass slide, coverslipped, and then viewed under a fluorescence microscope.

Criteria for Identifying Mitotic Figures

With hematoxylin staining, different stages of cell division in the M phase of the cell cycle were identified according to the following morphological criteria.18 Cells with visible, condensed chromosomes and an intact nuclear envelope were defined as being in the prophase. Cells with chromosomes aligned at a metaphase plate halfway between the poles and without a nuclear envelope were defined as being in the metaphase. Cells with separated chromatids being pulled toward the pole were defined as being in the anaphase. Cells with chromatids clustered at each pole, a contractile ring creating a cleavage furrow, and a reforming nuclear envelope were defined as being in the telophase.

Fluorescence Microscopy

A Nikon fluorescence microscope (Microphot-FX) was used to examine the specimens. LY fluorescence was studied with an excitation filter at 450 to 490 nm, a dichroic mirror at 510 nm, and a barrier filter at 520 nm. In en face preparations, the fluorescence intensity due to LY-LDL leakage was correlated with the occurrence of endothelial cell mitosis. Color slides of pictures were made with Kodak Ektachrome P500/1500 professional color reversal film 5020. The color slides were converted into black and white negative films and were printed.

The background level of fluorescence and the fluorescence intensity of LY-LDL spots were determined, and the size of LY-LDL spots were measured with an image processing system including a Panasonic WV-CD 50 camera (Panasonic Industrial Company, Secaucus, NJ), an EyeCom II image processor (Spatial Data Systems, Incorporated, Melbourne, FL), and a PDP 11-23 minicomputer (Digital Equipment Corporation, Maynard, MA) running under the RSX-11M operating system, which controls the image processor. Color slides were backlit using a light table and were scanned by the Panasonic camera. The resulting image signal was entered into the EyeCom II where it was digitized into a 640 × 480 array of pixels. The intensity values for each pixel ranged from a gray scale value of 0 for the black to 255 for the white of the light source. A computer program was developed to analyze the image. The analysis included a standard order of procedures including spatial calibration, image acquisition, thresholding, and profile extraction.

Results

Aortic pieces stained with hematoxylin were viewed with the fluorescence microscope. Figure 1 shows a fluorescence photomicrograph of an en face preparation of normal aortic endothelium after hematoxylin staining. The nuclei of individual endothelial cells were clearly identified without mitotic figures or fluorescence leaky
Figure 1. Fluorescence photomicrograph of an en face preparation of normal rat aortic endothelium after hematoxylin staining. The nuclei of individual endothelial cells are clearly seen. No mitotic figures or Lucifer yellow-low density lipoprotein fluorescence spots were found. × 2060

Figure 2. Fluorescence photomicrograph of an en face preparation of rat aortic endothelium after hematoxylin staining. A Lucifer yellow-low density lipoprotein (LY-LDL) fluorescence spot is shown associated with an endothelial cell undergoing mitosis in the prophase. This LY-LDL leaky spot is only slightly larger than the size of one endothelial cell. × 2600

Figure 3. Fluorescence photomicrograph of an en face preparation of rat thoracic aorta stained with hematoxylin. Note a dividing endothelial cell in the metaphase showing Lucifer yellow-low density lipoprotein fluorescence on part of its cell border. × 2060

Figure 4. Fluorescence photomicrograph of an en face preparation of rat thoracic aorta stained with hematoxylin showing the association of Lucifer yellow-low density lipoprotein leakage with a dividing endothelial cell in the anaphase. A mononuclear cell is seen adjacent to this mitotic cell. × 2060

either as spots (Figure 7) or as ellipsoidal rings (Figures 8 and 9A). Hematoxylin staining of the mitotic cells causes a partial masking of the fluorescence (Figure 9B) and a halo-like appearance (Figures 2 and 6).

The entire endothelial surface of each aortic piece was systematically scanned. The number of endothelial cells scanned was determined by dividing the endothelial surface area of each aortic segment by the average surface area of a single endothelial cell, which was found to be 520 μm². Table 1 shows the association of LY-LDL leakage with mitotic endothelial cells in the thoracic aortas of 10 rats. The average number of endothelial cells scanned in each rat was $2.19 \times 10^3$. Of the many endothelial cells scanned, the average mitotic frequency was only 0.034% (i.e., an average of 69 mitotic cells) per rat. Although the overall frequency of cell mitosis was quite low in the entire rat aortic endothelium, the dividing cells in different stages of the M phase showed a high incidence of junctional leakage to LY-LDL; on the average, 55 of 69 cells in the M phase were leaky (approximately 80%). These mitotic cells with LY-LDL leakage

spots. Figure 2 shows a fluorescence photomicrograph of an en face preparation of aortic endothelium after hematoxylin staining. An LY-LDL fluorescence spot was associated with an endothelial cell undergoing mitosis in the prophase. This leaky spot was only slightly larger than the size of one endothelial cell. A similar relationship between LY-LDL leakage and cell mitosis was also found in other phases of mitosis, for example, the metaphase (Figure 3), the anaphase (Figure 4), and the telophase (Figures 5 and 6). In these figures, the fluorescence of LY-LDL was particularly concentrated along the cellular junctions of mitotic cells and the cleavage furrow between two daughter cells.

The background fluorescence of the aortic endothelium was distinguishable from the fluorescence of LY-LDL as shown in Figures 7, 8, and 9A. Under the fluorescence microscope, the fluorescent LY-LDL can be more clearly identified in the aorta against the background. In the absence of hematoxylin, the LY-LDL fluorescence appears
Figure 5. Fluorescence photomicrograph of an en face preparation of rat aortic endothelium after hematoxylin staining. Fluorescence of Lucifer yellow-low density lipoprotein is seen along the cellular junction and cleavage furrow of a dividing endothelial cell in the late telophase. × 2080

Figure 7. Fluorescence photomicrograph of an en face preparation of rat thoracic aorta shows a Lucifer yellow-low density lipoprotein (LY-LDL) leaky spot before hematoxylin staining. The background fluorescence of the aortic endothelium is distinguishable from the fluorescence of LY-LDL. This spot has an area of 598 μm². The background level of the fluorescence is 130, and the fluorescence intensity of the LY-LDL spot is 210 (relative fluorescence intensity reading). Bar = 10 μm. × 2080

Figure 8. Fluorescence photomicrograph of an en face preparation of rat aortic endothelium after hematoxylin staining showing an endothelial cell undergoing mitosis in the late telophase with junctional leakage to Lucifer yellow-low density lipoprotein. × 2600

Figure 9. Fluorescence photomicrograph of an en face preparation of rat thoracic aorta. In the absence of hematoxylin staining, the Lucifer yellow-low density lipoprotein (LY-LDL) fluorescence appears as a ring. The background level of fluorescence is 128, and the fluorescence intensity of LY-LDL is 203 (relative reading). Bar = 10 μm. × 2600

accounted for 45% of all LY-LDL leaky sites. Table 2 shows a comparison between EBA and LY-LDL in terms of the relationship between mitotic cells and tracer leakage. Due to the larger molecular size of LY-LDL, the percentage of mitotic cells with junctional leakage was slightly lower than that for EBA; on the other hand, the mitotic cells were responsible for a higher proportion of the leakage spots for LY-LDL than for EBA.

Discussion

It is generally agreed that the vascular endothelium plays a key role in the initiation of the atherosclerotic processes. However, the initiation mechanisms, including the processes from lipid infiltration and monocyte recruitment to foam cell development, are still not completely understood. In the initial events of atherogenesis, the lipids have to be transported across the vascular endothelium at the sites that are prone to the development of atherosclerosis. The defective endothelium, either naturally occurring or experimentally induced, has been

suggested as the major site for lipid accumulation, but the pathway of lipid entry at the individual cell level still remains unclear.

In vivo studies have shown that the regions with an increased uptake of EBA conjugate (blue areas) are associated with a high endothelial cell turnover rate and an enhanced accumulation of lipids. In our recent study on normal rat aortas using EBA conjugate as a macromolecular tracer, we demonstrated that nearly all (99%) of endothelial cells in the M phase (as identified by hematoxylin staining of the dividing nuclei) exhibited EBA leakage 5 minutes after intravenous administration. These mitotic cells were responsible for 30% of all leaky spots. The observation that the mitotic figures and the cleavage
sites were usually located in the center of EBA leaky spots suggested that the leakage of albumin occurred through junctions around the mitotic cells and between daughter cells. These data provided the first experimental evidence in support of our hypothesis\(^1\) that macromolecular leakage occurs through open junctions in a specific time window of the cell cycle. During replication, the aortic endothelial cells continuously underwent morphological remodeling.\(^2\) The critical period of this remodeling appears to be the M phase, when the parent junctional protein strands are disrupted, cell shape undergoes its most dramatic change, and new junctions have to be formed after cleavage. Therefore, the endothelial cells undergoing mitosis would have open or poorly organized junctions through which macromolecules enter the arterial wall.

In our “leaky junction-cell turnover” hypothesis,\(^1\) the opening of a junction is assumed to occur gradually with time as the cells enter the M phase, from a normal spacing to a gap that is sufficiently wide to allow the passage of macromolecules as large as LDL. After the maintenance of this wide gap for a certain period of time in the M phase, the spacing then gradually returns to its normal dimension with the regeneration of new junctional complexes. According to this concept, the duration of junctional leakage to macromolecules and the fraction of leakage occurring during the M phase would vary inversely with the tracer molecular sizes. Our theoretical modeling\(^16,22,23\) indicates that a tracer such as horseradish peroxidase (HRP), which has a molecular diameter (about 5 nm) just borderline for its passage through a normal junction,\(^24\) can easily pass through a transiently open junction even when it is only slightly widened; thus the leakage would last for a fairly long period of time. For a larger tracer (e.g., the 6 nm albumin, which is essentially impermeable through a normal junction), leakage would not begin until the transiently open junction has widened to a significant extent; also, the time period for its passage would be more limited, and the size of the leaky spot would be smaller than that for HRP. For an even larger macromolecule (such as LDL with a diameter of 22 nm), leakage through an open junction would occur only when it is nearly maximally widened, and the duration of leakage would be confined to a narrow period (e.g., mainly the M phase) of the cell cycle. These considerations predict that the percentage of mitotic cells with transiently open junctions sufficiently wide for the passage of LDL would be less than that of albumin. Another prediction is that the fraction of the total number of leakage sites contributed by mitotic cells would be higher for LDL than albumin. Of course, beside the molecular size, factors such as electrical charge may also play a role in determining the duration and extent of leakage.

In the present study with LY-LDL as the macromolecular tracer, approximately 80% of mitotic cells exhibited junctional leakage to LY-LDL, which is substantially lower than the 99% found for EBA (Table 2). Also, these mitotic cells accounted for 45% of all cellular leakage sites, which is higher than the 30% found for EBA (Table 2). Although only 45% of the sites labeled by LY-LDL in this study were centered on cells in mitosis, our theoretical modeling\(^1\) predicts that this small fractional area of open clefts could quantitatively account for 50% to 100% of the focal increases in macromolecular permeability observed in large arteries, which in turn might lead to a significant accumulation of lipids and the formation of early foam cell lesions in regions prone to the development of atheroma. The observation that approximately 55% of the total LY-LDL leaky sites were associated with cells not morphologically identified as being in the M phase indicates that, in addition to intercellular gaps around mitotic cells, LDL leakage could also occur: 1) in time periods shortly preceding and following mitosis, 2) around dying endothelial cells that are being replaced by spreading of adjacent healthy endothelial cells, or 3) at sites of naturally occurring endothelial denudation. Since mitotic cells accounted for 45% of all LY-LDL leakage sites, they could be significant sites of LDL entry into the intima. Experiments to explore the nature of these nonmitotic cells with LDL leakage are in progress.
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Table 1. Association of Lucifer Yellow-Low Density Lipoprotein Leakage with Mitotic Endothelial Cells in Aortas of Rats

| Rat no. | No. cells scanned | Total LY-LDL leakage sites | No. cells found | Frequency (%) | No. cells with leakage | % Cells with leakage | % of Total leakage |
|---------|-------------------|----------------------------|-----------------|---------------|-----------------------|---------------------|--------------------|
| 1       | 2.08×10^6        | 138                        | 140             | 0.066         | 112                    | 80                  | 81                 |
| 2       | 3.55×10^6        | 52                         | 36              | 0.010         | 27                     | 75                  | 52                 |
| 3       | 2.90×10^6        | 141                        | 98              | 0.034         | 83                     | 85                  | 59                 |
| 4       | 1.09×10^6        | 110                        | 23              | 0.021         | 20                     | 87                  | 18                 |
| 5       | 2.10×10^6        | 94                         | 51              | 0.024         | 39                     | 76                  | 41                 |
| 6       | 2.28×10^6        | 150                        | 106             | 0.046         | 96                     | 61                  | 57                 |
| 7       | 1.70×10^6        | 115                        | 62              | 0.035         | 52                     | 94                  | 45                 |
| 8       | 2.22×10^6        | 185                        | 31              | 0.014         | 29                     | 94                  | 16                 |
| 9       | 3.08×10^6        | 152                        | 97              | 0.031         | 68                     | 70                  | 45                 |
| 10      | 0.89×10^6        | 92                         | 49              | 0.055         | 36                     | 73                  | 39                 |
| Mean    | 2.19×10^6        | 122.9                      | 69.3            | 0.034         | 55.2                   | 80.5                | 45.3               |
| SD      | 0.84×10^6        | 38.1                       | 38.7            | 0.018         | 30.7                   | 7.3                 | 19.2               |

LY-LDL = Lucifer yellow-low density lipoprotein. SD = standard deviation.

Table 2. Comparison of Percentage of Mitotic Cells with Tracer Leakage and Fraction of Tracer Leakage Sites with Mitotic Cell between EBA and LY-LDL

| Macromolecular % of mitotic cells | % of tracer leakage sites | % of mitotic cells | EBA    | 98.9 | 29.7 |
|-----------------------------------|---------------------------|-------------------|--------|------|------|
| LY-LDL                            |                           |                   | 80.5   | 45.3 |

EBA = Evans blue-albumin conjugate, LY-LDL = Lucifer yellow-low density lipoprotein.

In summary, the present study has provided experimental evidence in support of our "leaky junction-cell turnover" hypothesis that the transient opening of the junctions accompanying endothelial cell turnover provides pathways through which LDL enters the arterial wall to initiate lipid accumulation.

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