Europium Fluorescence to Visualize *N*-Butyl 2-Cyanoacrylate in Embolized Vessels of an Arteriovenous Malformation Swine Model

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BACKGROUND AND PURPOSE: **Standard tissue staining using the lipid dye Oil-Red-O has been previously applied to stain vessel specimens, which were embolized with a mixture of *n*-butyl 2-cyanoacrylate (NBCA) and oil (Lipiodol). That technique, however, results in nonspecific and nonquantitative staining that does not provide the necessary differentiation between NBCA and Lipiodol. We present an innovative staining procedure that quantifies NBCA within treated tissues.**

METHODS: **An arteriovenous malformation (AVM) model in swine was used to evaluate the polymerization characteristics of various ratios of Lipiodol/NBCA/glacial acetic acid (GAA) mixtures. To determine the depth of NBCA penetration within the AVM model and to characterize the polymerization patterns of various mixtures within the vessel, histologic cross- and longitudinal sections were prepared for microscopy. These paraffin-embedded tissue sections were stained with a europium aryl-β-diketone complex (TEC) to improve differentiation between NBCA and Lipiodol. Quantification of NBCA and Lipiodol within the lumen of rete cross-sections was accomplished using image analysis software to determine percent luminal area occluded by embolization.**

RESULTS: **Upon application of TEC, intense europium fluorescence was seen when the tissue samples were excited by low-power UV light (excitation at 365 nm; emission at 614 nm). The area of europium intensity within the lumen corresponded to NBCA concentration, and addition of GAA aided the NBCA distribution throughout the lumen without affecting fluorescence intensity. It was seen that NBCA could be easily differentiated from Lipiodol and that quantification could be readily performed on these sections because of the improved differentiation. For the case of a 50:50 (vol. %) mixture with an added 20 μL of GAA, luminal area distribution of Lipiodol, NBCA, and blood products was 42.6 ± 3.5%, 33.8 ± 5.7%, and 23.7 ±2.7%, respectively.**

CONCLUSION: **The rare earth metal europium, when added as a fluorescent chelate compound to histologic tissue sections, allowed for differentiation between NBCA and Lipiodol with good detail. These results have facilitated further characterization of NBCA polymerization for the use of AVM embolization.**

The most common liquid embolic agent used currently for endovascular treatment of arteriovenous malformations (AVMs) is *N*-butyl 2-cyanoacrylate (NBCA) or enbucrilate (1–3). In order to control the polymerization time of NBCA, as well as to opacify the mixture for angiographic visualization, an iodized poppyseed oil–based contrast agent (Lipiodol) is added to the NBCA. Glacial acetic acid (GAA) is also used to delay the polymerization time further by decreasing the pH of the mixture (4, 5).

Research efforts have focused on finding ways to improve quantification of the hemodynamic pa-

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rameters (ie, pressure, flow, and NBCA injection pressure) involved in AVM embolization, thus improving the reproducibility of successful outcomes for patients. Because the hemodynamic parameters involved have not been accurately quantified to date, NBCA embolization of AVMs is still considered a high-risk procedure. Improved characterization of embolization hemodynamics necessitates analysis of how deep NBCA penetrates into the AVM nidus. In addition, interaction of the NBCA and Lipiodol mixture with the vessel wall must be examined.

The approach chosen in this study was to examine histologic cross-sections of embolized tissue to assess the extent of embolization better. Harvested tissue obtained using an already well established AVM animal model was used (6–9). Standard techniques for processing and staining tissue containing cyanoacrylates have been described in the literature (10–12). However, previous attempts at chemical staining of NBCA-tissue composite sections performed using standard procedures have not provided the necessary differentiation between NBCA and Lipiodol under bright-field microscopy (4, 5).

Chelates containing fluorescent lanthanides (rare earth metals, such as europium) have been introduced as nonradioactive labels in immunocytochemistry and in situ hybridization (13, 14). The result has been development of simple and highly sensitive time-resolved fluoroimmunoassays with europium chelates to label proteins (15, 16), prostate-specific antigen (17), and other biomolecules (18–21). This work describes an improved method for staining embolized tissue through the application of europium. The application of europium fluorescence for the visualization of cyanoacrylate has been reported previously (22). The principle underlying this application involves production of a fluorescent europium tris chelate from the reaction of Eu^{3+} ions with an aryl-β-diketone. The europium aryl-β-diketone complex (TEC) is transferred into the interior of the cyanoacrylate from an aqueous methyl ethyl ketone solution, effectively "locking" the chelate inside the cyanoacrylate polymer.

Methods

Animal Experiments

Tissue used in this study consisted of swine retia harvested from acute and chronic AVM model studies. Acute embolization studies were performed surgically according to previously described methods (6), with chronic studies being based on the approach of Massoud (7, 8). Sources of supplies such as NBCA and Lipiodol, as well as the radiographic methods used in these studies, have also been described previously (23). All surgeries were performed in the School of Medicine and Biomedical Sciences at the State University of New York at Buffalo, under approval of the Institutional Animal Care and Use Committee.

Histologic Procedures

All chemicals and solvents used for tissue processing were purchased from the Aldrich Chemical Company (Milwaukee, WI).

Dissected retia for vessel histology were fixed in fresh buffered 3% formaldehyde for 2 hours (4°C), rinsed, dehydrated, paraffin-embedded, and radially sectioned (5 μm) onto poly-L-lysine-coated slides (Mt. Washington Scientific, Timonium, MD). Samples were obtained radially at predetermined angles spanning the entire rete from left to right of the ascending pharyngeal artery. Sections chosen for color staining were processed with the lipid dye Oil-Red-O, according to routine protocols.

Sections chosen for fluorescent staining were processed with a working solution of europium tris (thenoyltrifluoroacetone) or TEC. A 200-mL stock solution of TEC was first prepared by dissolving 0.25 g of europium trichloride hexahydrate in 100 mL absolute ethanol and combining this solution with 0.5 g of thenoyltrifluoroacetone dissolved in 100 mL of petroleum ether. The two solutions were combined by stirring for 5 minutes. Next, a working solution (100 mL) of TEC was prepared with 50 mL of stock solution added to 25 mL of petroleum ether and 25 mL of absolute ethanol.

Tissue samples chosen for TEC staining were processed in the following manner: three Coplin jars were arranged in sequence, with the first containing petroleum ether, the second containing TEC, and the third containing 100% methanol. A slide containing the embolized tissue sample was gently placed into the first jar for 1 minute to dissolve the paraffin completely. Next, the slide was removed and immediately transferred to the second jar (TEC) for 1 minute. Finally, the slide was removed and immediately transferred to the third jar (100% methanol) for 1 minute. The slide was then removed, air-dried, and mounted with 50% glycerol for subsequent microscopic analysis.

Microscopy and Image Analysis

All samples were examined at the Confocal Microscopy and 3-D Imaging Core Facility operated by the University. A Nikon FXA Automated Fluorescence Microscopy System equipped with 35 mm and 4-in × 5-in film cameras was used. The system also included a high-resolution, monochrome, computer-controlled, cooled CCD camera for fluorescence and color-bright field imaging. A DAPI filter cube was used for imaging samples under fluorescence. The excitation wavelength for TEC was at 365 nm, and the emission wavelength was at 614 nm (24). Microscope images were photographed with Kodak Elite Chrome color slide film (400 ASA).

Measurements of NBCA and Lipiodol within the lumen of rete cross-sections were accomplished by first tracing (with a computer mouse) the digitized images of the sections by using NIH Image software (Version 1.61) and subsequently determining percent luminal area as a ratio of corresponding pixel summation. Measurements were made in triplicate and were obtained from three different observers performing the tracing.

Results

High-resolution radiograms were obtained for various postembolized harvested retia in order to delineate the extent of NBCA penetration. Using the chronic AVM model, an 80:20 Lipiodol:NBCA ratio yielded proximal embolization with incomplete and inhomogeneous penetration of the rete (Fig 1). In addition, there appeared to be sputtered penetration beyond the midplane of the rete. Incomplete and inhomogeneous penetration of the rete was also the case when the ratio of Lipiodol:NBCA was changed to 50:50 (Fig 2). A more proximal occlusion than for the 80:20 case can be seen and inhomogeneity can be seen by the presence of voids along the injection path. Upon addition of 20
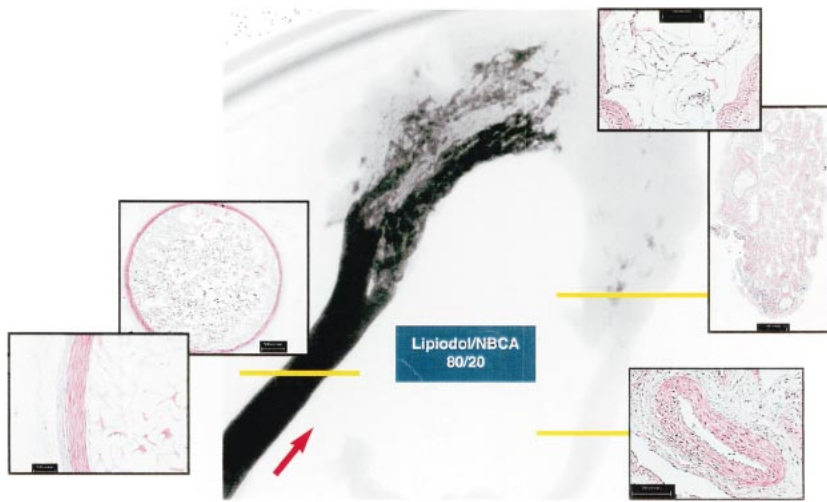
FIG 1. High-resolution radiogram and histologic stains of postembolized rete for chronic AVM model obtained using an 80:20 Lipiodol:NBCA ratio (volume %). Note *red arrow* indicates site and direction of glue injection during experiment. Three locations along the rete were selected for cross-sections, as indicated by the *yellow lines.* The *insets* represent the results of tissue staining at each indicated location. Scale bars are (clockwise, from upper right) 100, 2000, 100, 100, and 500 μm.

FIG 2. High-resolution radiogram and histologic stains of postembolized rete for chronic AVM model obtained using a 50:50 Lipiodol:NBCA ratio (volume %). Note *red arrow* indicates site and direction of glue injection during experiment. Three locations along the rete were selected for cross-sections, as indicated by the *yellow lines.* The *insets* represent the results of tissue staining at each indicated location. Scale bars are (clockwise, from upper right) 1000, 50, 100, 100, 1000, 100 and 2000 μm.
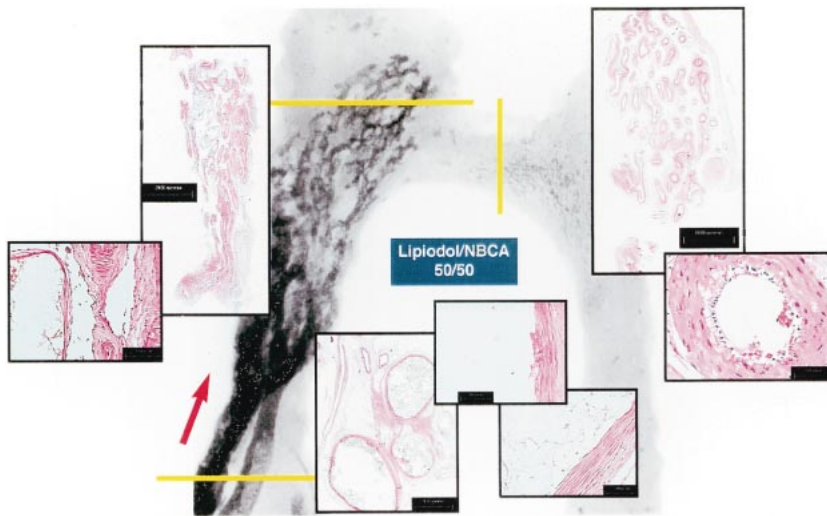

FIG 3. High-resolution radiogram and histologic stains of postembolized rete for chronic AVM model obtained using a 50:50 Lipiodol:NBCA ratio (volume %) with 20 μL of GAA added. Note *red arrow* indicates site and direction of glue injection during experiment. Two locations along the rete were selected for cross-sections, as indicated by the *yellow lines.* The *insets* represent the results of tissue staining at each indicated location. Magnifications are (clockwise, from upper right) 2000, 100, 200, 200, and 2000 μm.
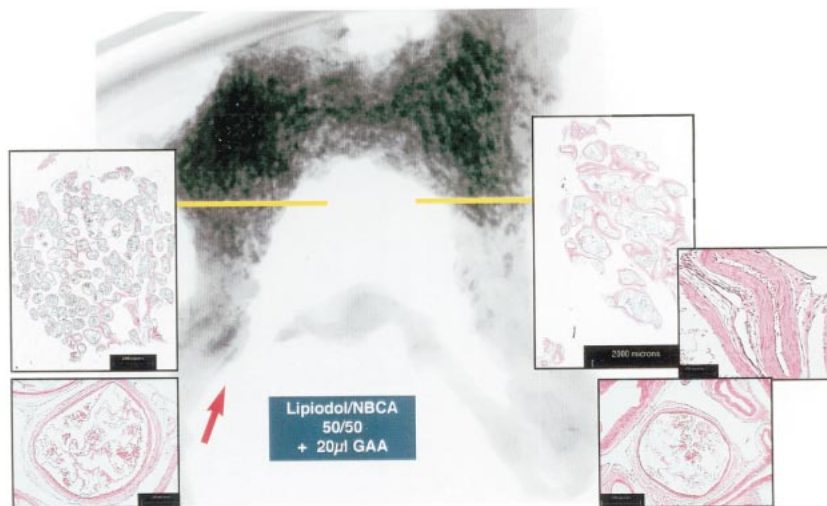
μL of GAA to a 50:50 mixture of Lipiodol:NBCA (1.8 mL total volume), a much more homogeneous penetration of the mixture was seen, as illustrated by an increased amount of radiopaque material within the contralateral ascending pharyngeal artery (Fig 3).

Embolized tissue cross-sections were evaluated histologically with respect to the degree of differentiation between NBCA and the surrounding tissue. This evaluation was done for all sections processed with the color stain Oil-Red-O. Representative samples are shown in Figures 1 through 3, wherein the yellow lines show various designated positions selected along the rete. At each position, the corresponding tissue cross-section is shown as stained with Oil-Red-O (inset). Because this lipid dye depends on the oleophyllic properties of NBCA, regions of tissue containing NBCA manifest a pink-red color. However, two difficulties arose with using this type of color stain. First, it became difficult to distinguish between tissue, Lipiodol, and any residual blood/blood products. Secondly, various shades of pink-red were present in the sections as shown in Figures 1 through 3. It was not possible to determine whether these different shades of color were because of nonuniformity in tissue staining or if color intensity was directly proportional to the concentration of NBCA within a particular region of tissue. Owing to these qualitative difficulties, a more quantitative method for determining NBCA concentration within embolized tissue was sought using europium fluorescence.

Several control experiments were performed first in order to examine the selective binding of europium to NBCA within a tissue section. Staining experiments were designed to examine europium binding to NBCA solely on the presence (or absence) of a fluorescent signal when sections were exposed to UV light under microscopy. This binding was examined under both dried and undried conditions. The dried state simulated actual tissue preparation conditions, because NBCA rapidly polymerizes after injection into the vasculature. Application of europium to undried samples was used to determine if NBCA polymerization affects fluorescence to any degree. In order to examine europium binding to NBCA in a dried state, 15 μL of NBCA was allowed to air-dry onto a glass slide 20 minutes prior to application of 15 μL TEC onto the NBCA. To examine NBCA in an undried state, TEC was immediately applied onto the NBCA droplet. The volume (15 μL) for both NBCA and TEC was determined after numerous trials to be an optimal volume for microscopic slide preparation. These control experiments subsequently demonstrated that europium did bind to NBCA, confirming the findings of Wilkinson and Watkin (22). Interestingly, binding was observed for both undried and dried states of NBCA.

Control experiments were also performed to determine any europium binding to Lipiodol. When a 15-μL volume of Lipiodol was viewed under UV light with and without europium staining, no distinction could be made. Further control experiments were designed to determine europium's binding behavior to the mixture of Lipiodol and NBCA. When staining both with and without europium, it was found that fluorescence was seen exclusively in the sample containing europium. The end result of these control experiments led to the observation that europium selectively binds only to the NBCA in the NBCA-Lipiodol mixture.

When paraffin-embedded cross-sections of rete tissue were examined under UV light, several interesting results were observed. Figure 4 shows a section through a rete embolized with a Lipiodol:NBCA mixture of 80:20. The left panel shows that an exogenous material (presumably polymerized NBCA) took the form of irregular, interwoven folds within the lumen, and that portions of this material adhered to the vessel wall. When tissue was processed with europium (TEC), an intense fluorescence was seen (right panel), which was absent in the europium-free tissue (left panel). This result suggests that a europium tris chelate can be used to target NBCA in embolized tissue sections. The NBCA fluorescence was seen as orange in color, appearing as a distinctly intense band of uniform thickness and coursing the same irregular, interwoven pattern as that determined to be polymerized NBCA (left panel of Figure 4). Lipiodol, in contrast, appeared dark (or opaque) in Figure 4 (both panels). This was expected, because the control experiments demonstrated that adding europium to Lipiodol did not induce any fluorescence. Finally, material (presumably blood and/or blood products) was observed within some of the folds formed by the polymerized NBCA. Continuous flow during embolization entraps blood and/or blood products upon polymerization of NBCA. As seen in a lower magnification photograph for the 80:20 case (Figure 5), regions within the vessel lumen can be readily distinguished from Lipiodol because of the europium fluorescence. Figure 5 also illustrates more clearly that NBCA tends to adhere to the vessel wall.

When the ratio of Lipiodol:NBCA was changed from 80:20 to 50:50, it was expected that NBCA would be present within the lumen at a higher concentration. Cross-sections processed with europium confirmed this prediction (see Figures 5 and 6). The higher concentration of NBCA produced a thicker band of europium fluorescence (Figure 6), whereas the lower concentration showed a narrower band (Figure 5). This demonstrates that, within the cross section of vessel lumen, the area of europium intensity corresponds to NBCA concentration. The tissue section in Figure 6 also showed some stripping of the endothelium. Figure 7 shows a section through a blood vessel from a rete that was embolized with a mixture composed of 0.9 mL of Lipiodol and 0.9 mL of NBCA, with the addition of 20 μL GAA (intended to delay NBCA polymerization). Comparison of Figure 6 with Figure 7
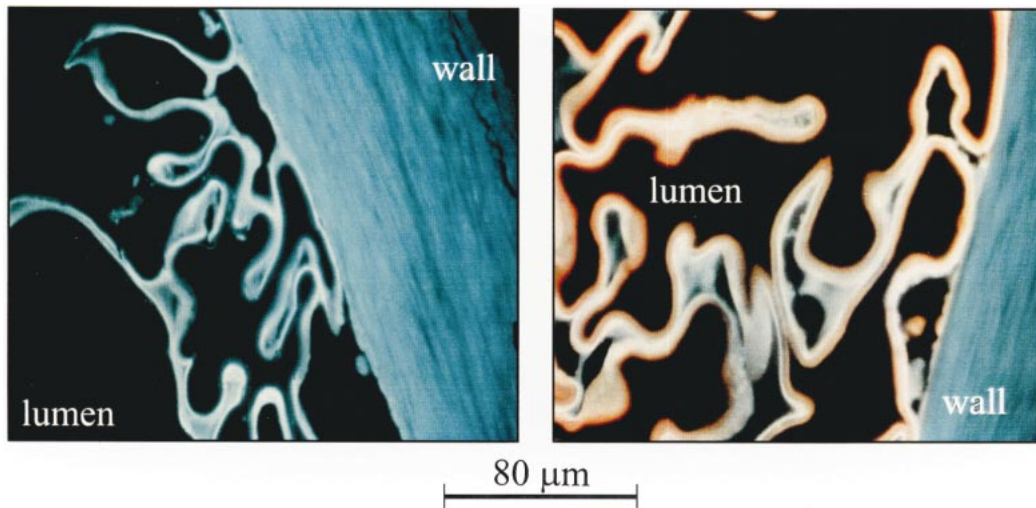
FIG 4. Paraffin-embedded rete tissue after europium emission observed at 614 nm for an 80:20 Lipiodol:NBCA ratio. Cross-sections were processed in the absence of TEC (*left panel*) and with TEC (*right panel*). Positions of the vessel wall and lumen are indicated. Scale bar distance is as shown.
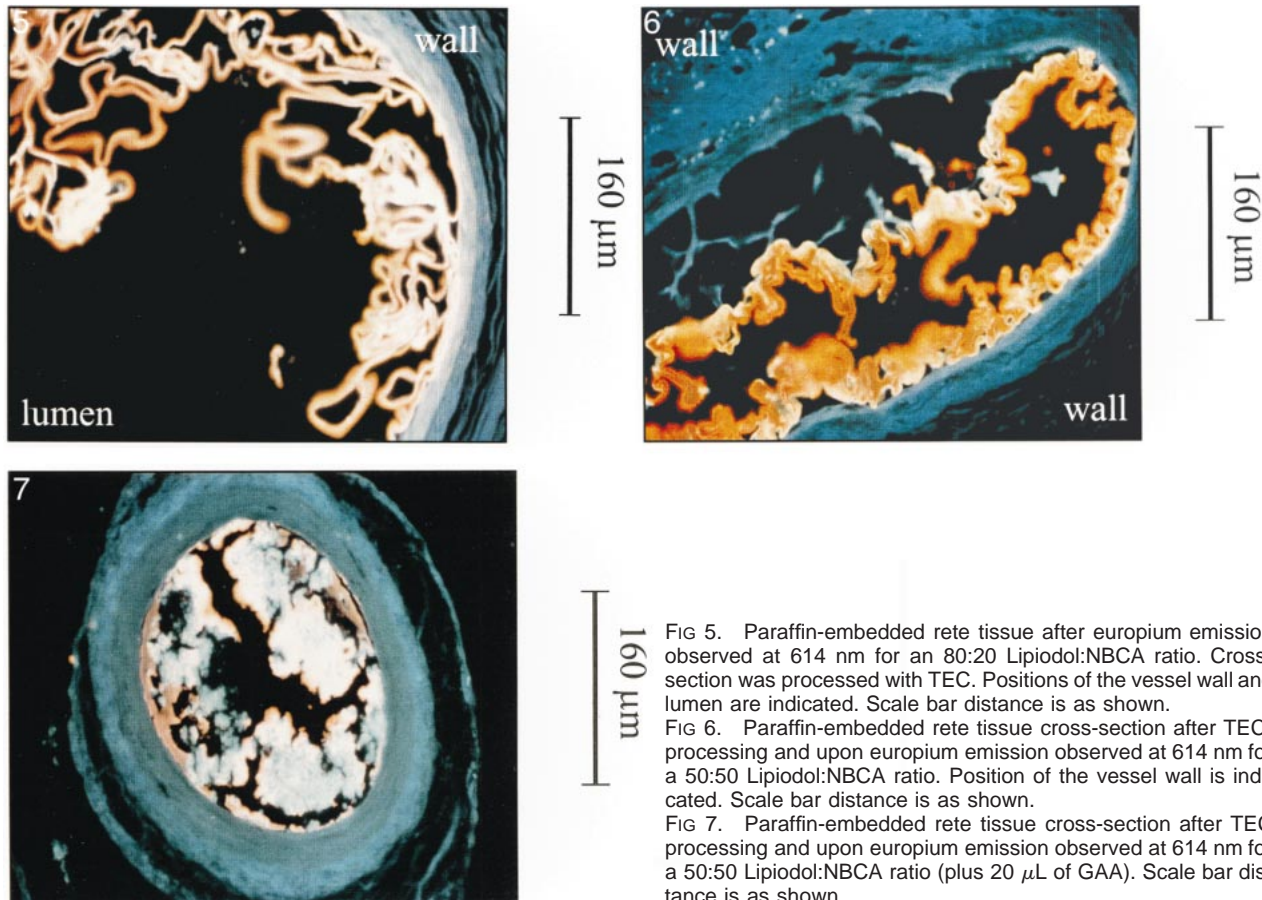

FIG 5. Paraffin-embedded rete tissue after europium emission observed at 614 nm for an 80:20 Lipiodol:NBCA ratio. Cross-section was processed with TEC. Positions of the vessel wall and lumen are indicated. Scale bar distance is as shown.

FIG 6. Paraffin-embedded rete tissue cross-section after TEC-processing and upon europium emission observed at 614 nm for a 50:50 Lipiodol:NBCA ratio. Position of the vessel wall is indicated. Scale bar distance is as shown.

FIG 7. Paraffin-embedded rete tissue cross-section after TEC processing and upon europium emission observed at 614 nm for a 50:50 Lipiodol:NBCA ratio (plus 20 μL of GAA). Scale bar distance is as shown.

demonstrates that the addition of GAA facilitates NBCA distribution throughout the lumen, preventing its concentration in a narrow band adjacent to the wall. However, the addition of such a small quantity of GAA had no effect on the intensity of fluorescence.

Discussion

Histopathologic studies were performed on autopsy tissue of swine rete. Both acute and chronic animal models were used in these studies to create conditions that better mimic human AVMs. Histologic comparison of the chronic versus acute model
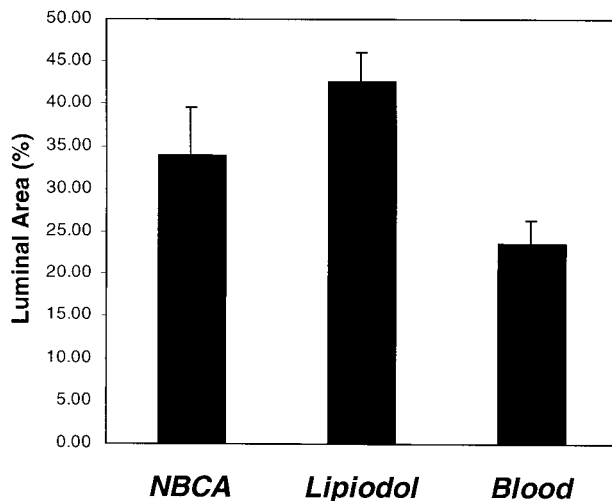
FIG 8. Distribution of the various components comprising the glue-oil mixture for an NBCA:Lipiodol ratio of 50:50 (with 20 μL of GAA added), as measured by image analysis of the TEC-processed tissue cross-section (see Figure 7).

can help determine if vascular wall modifications (such as ectasia) could be induced experimentally through, for example, weakening of the vascular wall or the creation of collagen voids.

Radiograms of excised embolized retia (Figures 1 through 3) indicate the depth of penetration of the Lipiodol-NBCA mixture. However, the histologic results with Oil-Red-O (as seen by the various insets in each figure) only serve to qualitatively confirm these radiographic results.

In order to quantify the degree of occlusion by NBCA within the vessel, europium staining of tissue was used. The results of the experiments shown in Figures 4 through 7 concur with the findings of Wilkinson and Watkin (22) in that intense fluorescence was seen when a europium tris chelate (aryl-β-diketone complex) was excited by UV light. As with that study, minimal background fluorescence was observed when examining the tissue cross-sections.

The excellent contrast provided by TEC (Figure 4, right panel) was not affected by the enhanced autofluorescence normally caused by formaldehyde fixative (Figure 4, left panel). A similar finding was noted by Seveus et al (25), who examined europium chelates and their effect on enhanced autofluorescence caused by glutaraldehyde.

A quantitative determination using image analysis was made for the 50:50 (plus 20 μL GAA) mixture seen in Figure 7. This subsequently allowed for calculation as to what (in terms of area) constituted NBCA (and alternatively, Lipiodol) within the cross-section. The result of this analysis (Figure 8) showed that, within statistical error, calculated NBCA and Lipiodol concentrations matched the respective concentrations initially used during the actual experiment.

Histologic examination of the stained retia (using either Oil-Red-O or europium) revealed that, for both stains, NBCA does not fill the entire lumen of the vessel. Further examination of Lipiodol in the lumen also shows different visual characteristics with the two staining techniques. It appears that pockets of NBCA surrounded by Lipiodol are formed with Oil-Red-O staining. Conversely, when europium staining is used, it seems that pockets of Lipiodol encapsulated by NBCA are formed. Because this improved staining technique allows for better visualization of the Lipiodol-NBCA interface, it is therefore hypothesized that Lipiodol becomes sequestered by the NBCA upon polymerization. This point is substantiated by the interesting clinical observation that, with time, the NBCA-Lipiodol mixture used in AVM-embolized patients decreases in radiopacity. Thus, a decrease of Lipiodol concentration within the glue mixture is suggested rather than a reabsorption by macrophages, as previously postulated.

These results seen in paraffin sections confirm the findings of Stoesslein et al (26), who observed that the NBCA and oil really do not form a compound, but rather are mixture components that can be selectively identified. The oil, visually distinguished by spaces within the lumen, is usually seen within the inside diameter of the section, whereas the NBCA is situated along the periphery. From a hemodynamic perspective, as the injected embolic mixture travels through the vessel, the velocity is greatest at the centerline and zero near the vessel wall (if a no-slip boundary condition is assumed). This phenomenon facilitates NBCA polymerization near the vessel wall. It also explains the histologic results, which show that NBCA has a higher tendency to adhere to the vessel wall. Only small amounts of NBCA are observed in the vicinity of the vessel centerline because, intuitively, NBCA would be more likely to be displaced toward the vessel wall as flow progresses (Figures 5 through 7). Thus, investigators recommend a wedge position of the microcatheter during NBCA injection or asystole, which ensures a flow reduction or arrest and a more homogeneous distribution of the glue within the nidus (27, 28).

Another observation in these experiments involves the conservation of tissue endothelium upon processing with europium. The photograph in Figure 6 indicates that some regions within a cross-section demonstrated an intact endothelium, whereas other regions showed stripping of the endothelium. In this study, whether such stripping reflects the toxic effects of NBCA polymerization or mechanical artifact in histologic sectioning remains unknown. Consequently, the NBCA polymer provides a subocclusive thrombogenic matrix that causes partial or complete thrombosis. Acute effects of embolization showed multiple arteries and arterioles within the system containing an irregular meshwork of polymerized NBCA, thus confirming the observations by Kish et al (29). This observation may explain why, under certain circumstances,

although NBCA is used, a recanalization of an embolized AVM may occur (30).

To our knowledge, this is the first study using europium in the development of a fluorescent staining technique for postembolized cerebrovascular tissue (31 [patent pending]). Europium fluorescence as a technique for distinguishing NBCA from Lipiodol and blood/blood products in embolized tissue was found to give satisfactory results. Issues that require further investigation include examination of the sequestering behavior of the NBCA polymerization process as well as determination of endothelial integrity (perhaps through examination of inflammatory processes).

Conclusion

The use of europium staining allows for better characterization of the Lipiodol-NBCA mixture behavior after embolization compared with Oil-Red-O staining. When europium was added as a fluorescent chelate compound to embolized tissue, the result was an ability to selectively detect NBCA with good detail. This method does not rely on qualitative detection of NBCA, but rather it allows for subsequent quantitative measurement through image analysis. The results of this study should lead to more precise evaluation of the parameters that characterize the polymerization of embolization that improve the technique of glue embolization for an AVM.

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