Multiple Labeling in Electron Microscopy: Its Application in Cardiovascular Research

M. M. H. Marijanowski
*University of Amsterdam*

P. Teeling
*University of Amsterdam*

K. P. Dingemans
*University of Amsterdam*

A. E. Becker
*University of Amsterdam*

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MULTIPLE LABELING IN ELECTRON MICROSCOPY: ITS APPLICATION IN CARDIOVASCULAR RESEARCH

M.M.H. Marijanowski, P. Teeling, K.P. Dingemans, and A.E. Becker*

Department of Cardiovascular Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

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Abstract

The heart is a muscular pump kept together by a network of extracellular matrix components. An increase in collagens, as in chronic congestive heart failure (CHF), is thought to have a negative effect on cardiac compliance and, thus, on the clinical condition. Conventional electron microscopy allows for the study of cellular and extracellular components and scanning electron microscopy (SEM) can put these structures in three-dimensional perspective. However, in order to study extracellular matrix components in relation to cells, immunoelectron microscopy is superior. We have used this technique in our studies on heart failure. Heart specimens were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in sodium cacodylate buffer, dehydrated by the method of progressive lowering of temperature and embedded in LR Gold plastic. Immunolabeling could be achieved with different sized gold-conjugated secondary antibodies or protein-A gold conjugates. Depending on the objective, ultra small gold (USG) conjugates or a regular probe size can be used. Labeling efficiency could be increased by bridging antibodies. The double and triple staining procedures were based on single staining methods using one- and two-face labeling. The choice of antibodies and gold conjugates depended on the objectives. Immunelectron microscopy, using multiple labeling, allowed a detailed study of the organization of the extracellular matrix and its relationship with cardiac myocytes. This may prove to be a useful tool for the study of chronic heart failure.

Key Words: Immunoelectron microscopy, double-labeling, triple-labeling, collagen type I, collagen type III.

*Address for correspondence:
Anton E. Becker
Department of Cardiovascular Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
Telephone number: (+31) 20 5665646
FAX number: (+31) 20 6914738

Introduction

The extracellular matrix plays an important role in the regulation of tissue function and a close relation exists between the various proteins and cells. Collagens are a source of strength to the tissue, elastin and proteoglycans are essential to matrix resiliency, and the structural glycoproteins help to create tissue cohesive­ness. The extracellular matrix of the heart is comprised of various components which are organized into a three-dimensional network (Caulfield and Borg, 1979; Borg and Caulfield, 1981; Borg et al., 1982; Robinson et al., 1983). The epimysium is defined as the sheath of connective tissue that surrounds the entire muscle; endomysium is the connective tissue that surrounds and interconnects cells; perimysium connects epimysium to endomysium and surrounds groups of myocytes. The form and distribution of the connective tissue of the heart is such that it may play an important role in the elastic properties of the ventricles (Borg et al., 1981). The collagen network in particular is thought to play an important role in maintaining the integrity of the heart muscle. The collagen fibers provide a scaffolding that supports muscle cells and blood vessels. They act as lateral connections between cells and muscle bundles and by virtue of this architecture govern the delivery of force generated by the myocytes. In this setting the collagens determine myocardial stiffness (Weber, 1989). The major collagens are collagen type I and type III, which possess different physical properties: collagen type I provides rigidity whereas collagen type III contributes to elasticity. Hence, the ratio of these two types of collagen is important. In hearts of patients with chronic congestive heart failure (CHF) there is an increase in extracellular matrix components (Heneghan et al., 1991; Schaper and Speiser, 1992; Yoshikane et al., 1992; Marijanowski et al., 1995). Moreover, the ratio between collagen type I and type III is changed in favor of collagen type I.

Light and conventional electron microscopy have established these observations. Scanning electron microscopy (SEM) has revealed the three-dimensional relation between matrix components, cardiac myocytes and connective tissue cells. However, the exact identification...
of components and cells is possible only by immunolabeling. Because of the functional implications a detailed study of the changes in extracellular matrix components in hearts of patients with chronic CHF may contribute to an understanding of the progressive and often irreversible nature of the disease.

This article discusses the technical aspects of multiple labeling techniques, with an emphasis on the techniques specifically applied in our studies on chronic heart failure.

Multiple Labeling Techniques in Immunoelectron Microscopy

Many monoclonal antibodies will not recognize an epitope in plastic-embedded sections because fixation and embedding may alter the tertiary structure of the epitope. In this case immunolabeling on cryosections is an option, although the morphology is less well preserved and it takes more effort to obtain good quality contrast. The present review will focus on plastic post-embedding procedures, instead of pre-embedding procedures, because the antigen detection using gold conjugates and gold-conjugated antibodies generally gives better results compared to detection with enzymes. The gold particle is smaller and more discrete than the enzyme precipitate in the tissue and, therefore, allows for a more precise localization. Furthermore, once the tissue is embedded in plastic the antigenicity is preserved well enough to use the tissue specimen for detection of several antigens. This contrasts with pre-embedding procedures that are done for the detection of one antigen only and do not allow further researches for other epitopes in the same tissue block.

Fixation, dehydration and embedding in plastic

The choice of fixation is dependent on the balance between optimal antigen detection and acceptable preservation of morphology (Craig and Goodchild, 1982). A fixation with 4% paraformaldehyde and 0.1% glutaraldehyde in sodium cacodylate buffer (pH 7.4) for about 1 hour at 4°C often meets both demands. The most propitious procedure to overcome loss of antigenicity is either embedding in plastic or processing for cryoultramicrotomy immediately after fixation. However, it is possible to store tissue specimens in 0.5% buffered paraformaldehyde at 4°C for several weeks without complete loss of antigenicity; a feature which depends on the nature of the antigen. Another way of storing the specimens is to immerse the tissue blocks in 2.3 M sucrose after washing them in 50 mM glycine in phosphate buffered saline (PBS) at 4°C. These tissue specimens are then used for cryoultramicrotomy or for freeze substitution.

Dehydration of the tissue samples is done either by using progressive lowering of temperature (PLT) or by freeze substitution. Ethanol, methanol, acetone, dimethylformamide or ethylene-glycol can be used as the dehydrating agents (Quintana, 1994). The PLT method is based on a lowering of the temperature during dehydration and a subsequent impregnation with the appropriate plastic (see below). Dehydration by freeze substitution requires cryoprotection of the tissue specimen by immersion in 2.3 M sucrose at 4°C after which the tissue specimens are frozen in liquid nitrogen and dehydrated in methanol containing 0.5% uranyl acetate at -80°C. The temperature is slowly raised to -45°C, after which the tissue is impregnated with the plastic.

Several plastics are available for immuno-electron microscopy, such as Lowicryl K4M, HM20, LR White, and LR Gold. Both Lowicryl K4M and HM20 are made of acrylate and methacrylate and are still liquid at very low temperatures. K4M is a polar (hydrophilic) plastic and can be used at -30°C. HM20 is apolar (hydrophobic) and can be used at -50°C. Polymerization of both plastics is carried out under ultraviolet light at -30°C and -50°C, respectively. LR Gold is a plastic based on acrylate with hydrophilic properties and stays liquid at -20°C. Polymerization is also done under ultraviolet light but at -20°C. The polymerization at low temperatures under ultraviolet light is followed by further polymerization at room temperature for 48 to 72 hours.

The plastics differ in cutting qualities, preservation of morphology and antigenicity (Herken et al., 1988a; Herken et al., 1988b; Kann and Fouquet, 1989; Benichou et al., 1990; de Mesy Jensen and di Sant'Agnese, 1992; Hamilton et al., 1992; Migheli et al., 1992; Robertson et al., 1992; Kasper and Migheli, 1993). The published results regarding LR White and LR Gold are contradictory. Most authors favor LR Gold, although LR White is usually preferred when membrane contrast is important (Taatjes et al., 1994).

Sectioning

Tissue sections of 50 to 70 nm thickness are cut and collected on Formvar-coated 50 mesh copper grids. When the detection with the primary antibody is performed with ultra-small gold (USG) conjugates and visualization using silver enhancement, the tissue sections must be collected on Formvar-coated 50 mesh nickel grids. In case of multiple immunolabeling on both sides of the grid, uncoated (200-700 mesh hexagonal) grids are used.

Immunolabeling and detection

The following paragraphs are based on the procedures that we use. Only a general outline of the methods can be provided, since detailed instructions are highly
Multiple labeling in electron microscopy

Figure 1: Immunolabeling for actin in smooth muscle cells from aortic tissue. A. Labeling with anti-actin antibody (clone 1A4) and protein-A/10 nm. B. Labeling with the same primary anti-actin antibody, a rabbit anti-mouse secondary antibody and finally protein-A/10 nm. C and D. The primary antibody is anti-actin (clone HHF35). C shows direct labeling of the primary antibody with protein-A/10 nm and D using a bridging antibody. Bar=0.5 µm.

dependent on variables like tissue and antigen characteristics, tissue fixation and the plastic used for embedding. We usually start by using single-immunostaining to identify the staining characteristics of the antigens to be studied. This allows for the proper sequence in the multiple staining procedures. This procedure also appears to be in line with that promoted by Bendayan et al. (1987), who stated that no single procedure can be recommended as the best approach in cytochemistry.

The detection of the primary antibody can be done with either gold-conjugated secondary antibodies, USG conjugates or protein-A or protein-G gold conjugates.

For the gold-conjugated secondary antibodies, several sizes of gold particles are available. USG conjugates have a probe size smaller than 1 nm, which comes so close to the resolution of the electron microscope that it starts to interfere with adequate imaging (Shimizu et al., 1992). Enlargement of the probe (sizes larger than 40 nm are possible) with silver enhancement is therefore necessary. The use of USG conjugates is functional because of the high labeling efficiency of the small probe and a good overview at low magnification because of the large probe size after silver enhancement. The silver enhancement procedure according to Danscher (1981) gives reproducible results and the amplification time corresponds well with the total enlargement of the probe. Protein-A or -G gold conjugates react with the immunoglobulin (Ig)G portion of the primary antibody and have different affinities for several species (Bendayan, 1982; Roth, 1982; Silver and Hearn, 1987; Holm et al., 1988; Roth, 1989; Roth et al., 1989). Both have a high affinity for rabbit and guinea pig IgG. However, with antibodies raised in goats or mice and particularly with mouse monoclonal antibodies, protein-G gold conjugates yield intense and specific labeling, whereas protein-A gold conjugates yield more variable results (Bendayan and Garzon, 1988). In the case of multiple immunolabeling procedures, a brief treatment with 1% glutaraldehyde in PBS after the incubation with protein-A or protein-G is necessary to inhibit the antibody complex.

For all three options, i.e., protein-A, protein-G gold conjugates and gold-conjugated secondary antibodies, the size of the gold conjugate influences the labeling efficiency, because steric hindrance increases with probe size which in turn results in a decreased labeling (Yokota, 1988).
Table 1. Progressive lowering of temperature dehydration and embedding protocol.

1. Cut tissue in blocks not larger than 2 mm³

2. Dehydrate for 10 min.
   in ethanol 30% 4°C 0°C 0°C

3. Dehydrate for 10 min.
   in ethanol 50% 0°C -10°C -10°C

4. Dehydrate for 30 min.
   in ethanol 70% -10°C -20°C -20°C

5. Dehydrate for 30 min.
   in ethanol 90% -20°C -30°C -30°C

6. Dehydrate for 30 min.
   in ethanol 100% -20°C -30°C -50°C

7. Dehydrate for 30 min.
   in ethanol 100% -20°C -30°C -50°C

8. Impregnate for 1 h.
   in ethanol:plastic 1:1 -20°C -30°C -50°C

9. Impregnate for 1 h.
   in ethanol:plastic 1:2 -20°C -30°C -50°C

10. Impregnate in pure plastic overnight
    at appropriate temperature.

11. Replace plastic twice.

12. Place tissue blocks in gelatine capsules,
    fill with appropriate plastic, seal
    and polymerize at appropriate temperature
    under UV light.**

*Gold-conjugated antibodies:

Goat anti-rabbit gold conjugate with sizes ranging from 5 to 20 nm.
Goat anti-mouse gold conjugate with sizes ranging from 5 to 20 nm.
Goat anti-rabbit or goat anti-mouse ultra-small (<1 nm) gold conjugate.
or: Protein-A gold conjugate with sizes ranging from 5 to 20 nm.

All steps are performed on a clean sheath of Paraﬁlm with the grids, section face downwards, on a 30-50 µl droplet. During the above labeling protocol the sections should not be allowed to dry since this may give rise to non-specific staining and contamination (dirt) of the contrasted section.

Protein-A binds strongly to rabbit, guinea pig and human IgG, with mouse IgGs it binds with different aﬃnity. If speciﬁc antibodies with weak binding capacity for protein-A are used (sheep, goat and some mouse IgGs) or when multiplication of the gold signal is desired, a bridging antibody (rabbit anti-goat or mouse or swine anti-rabbit) should be included. The incubation time is 30 minutes.

Detection of two epitopes

The mode of detection is dependent on the primary antibodies used. The detection of two epitopes with primary antibodies from diﬀerent species is performed by diluting both antibodies in a mixture. The same is valid for the corresponding secondary antibodies (using diﬀerent probe sizes). In this case the standard protocol for single-staining can be used.
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Figure 2: Immunolabeling of actin in aortic tissue using the same primary anti-elastin antibody (clone 1A4). The secondary labeling is a rabbit anti-goat antibody conjugated with 6 nm (A) and 12 nm (B) gold. Bar=0.5 \mu m.

The protocol for double staining is used when one of the gold conjugates is protein-A. Protein-A will react with the second primary antibody and therefore an inhibition step is necessary. The protocol for double staining is also used when the primary antibodies are from the same species. A one-face method can be performed using a combination of protein-A or protein-G and a gold-conjugated secondary antibody. The use of different sized gold conjugates of the same species is possible when a two-face method is used.

For the two-face immunolabeling method, sections are collected on uncoated 200-700 mesh hexagonal copper grids. A single staining is performed on either side of the grid, using two different-sized gold conjugates to detect the primary antibody. After labeling the grid is coated with Formvar on the side where the sections have been collected to prevent damage by the electron beam.

Another possibility is detection of the first epitope with USG and enhancement of the probe. The enlargement of the USG will mask the epitope-antibody complex. This method cannot be used when both epitopes are situated at a short distance from each other. The enlargement of the USG can cover both epitopes, or the detection of the second epitope is not possible because of steric hindrance. For this case, the two-face labeling method has to be used.

Detection of three epitopes

In general, the protocol for the double staining can be used. The choice of either a one-face or a two-face detection method depends on the primary antibodies used.

Background reduction

The use of BSA-C (Aurion, Wageningen, The Netherlands) will prevent background. BSA-C is an acetylated and linearized form of bovine serum albumin and competes with the negative charge of the gold particles, thus preventing charge-determined background. Furthermore, the hydrophobic binding sites which are introduced in many plastics are blocked.

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Table 3. Double staining with gold conjugated secondary antibody and primary antibodies from the same species

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Collect sections on Formvar-coated grids</td>
</tr>
<tr>
<td>2.</td>
<td>Block free aldehyde groups with PBS/glycine 50 mM</td>
</tr>
<tr>
<td>3.</td>
<td>Wash with PBS/BSA-C (0.2%)</td>
</tr>
<tr>
<td>4.</td>
<td>Incubate with primary antibody diluted in PBS/BSA-C (0.2%)</td>
</tr>
<tr>
<td>5.</td>
<td>Wash with PBS/BSA-C (0.2%)</td>
</tr>
<tr>
<td>6.</td>
<td>Incubate with gold conjugated secondary antibody*</td>
</tr>
<tr>
<td>7.</td>
<td>Wash with PBS</td>
</tr>
<tr>
<td>8.</td>
<td>Fix/inhibit the antibody complex with 1% GA in PBS</td>
</tr>
<tr>
<td>9.</td>
<td>Wash with PBS</td>
</tr>
<tr>
<td>10.</td>
<td>Block free aldehyde groups with PBS/Glycine 50 mM</td>
</tr>
<tr>
<td>11.</td>
<td>Wash with PBS/BSA-C (0.2%)</td>
</tr>
<tr>
<td>12.</td>
<td>Incubate with secondary antibody diluted in PBS/BSA-C (0.2%)</td>
</tr>
<tr>
<td>13.</td>
<td>Wash with PBS/BSA-C (0.2%)</td>
</tr>
<tr>
<td>14.</td>
<td>Incubate with different sized gold conjugated secondary antibody*</td>
</tr>
<tr>
<td>15.</td>
<td>Wash in PBS</td>
</tr>
<tr>
<td>16.</td>
<td>Jetwash with distilled water and dry at room temperature</td>
</tr>
<tr>
<td>17.</td>
<td>Contrast lightly with uranyl acetate and lead citrate</td>
</tr>
</tbody>
</table>

*Gold-conjugated antibodies (see Table 2).
Figure 3: Immuno-double labeling on human heart tissue. A. Immunolabeling of a vessel wall. The Weibel-Palade bodies (indicated with an arrow) are stained with anti-vWF antibody using protein-A/10 nm. The lectins are stained with UEA-1 and a protein-A gold/15 nm. Bar represents 0.5 µm. B. Staining of the extracellular matrix of the heart. Collagen type III is stained with a secondary rabbit anti-goat antibody detected with 10 nm gold and collagen type VI with 15 nm gold. A two-face method is used. Bar represents 0.5 µm. C. Staining of collagen type VI and elastin in the extracellular matrix of a heart. Collagen type VI and elastin antibodies are labeled with protein-A/10 and 15 nm, respectively. Bar=0.5 µm.

Materials and Methods

In this section, we will outline the specific procedures used in our studies in cardiovascular research.

Tissue sampling

All labeling experiments shown in this paper are performed on aortic and heart tissue specimens.

The hearts used were obtained from patients with chronic CHF on the basis of dilated cardiomyopathy (DCM, n=4) and ischemic heart disease (IHD, n=4), clinically all in the New York Heart Association classes IV and V, and collected as cardiac explants or at autopsy. The autopsy hearts all became available within 10 hours after death. Heart tissue from adult patients (n=4) who died of noncardiovascular-related diseases, served as reference.

The hearts were cut perpendicular to the long axis of the left ventricle. Tissue samples were obtained from the left ventricular wall at the level of the base of the papillary muscles. Aortic tissue was obtained from the same hearts.

The tissue specimens were cut into small pieces (1-2 mm³), fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in sodium cacodylate buffer (pH 7.4), embedded in LR Gold plastic after dehydration in ethanol using the PLT method. The protocol for the dehydration and embedding that we have used is shown in Table 1.

We prefer the use of LR Gold plastic because we have most experience with this particular plastic and, furthermore, it fitted our research objectives well.

Immunolabeling procedures

Our protocol for the detection of a single epitope is shown in Table 2 and that for the detection of two epitopes in Table 3.

All primary antibodies used in this study are listed in Table 4. Protein-A conjugated with 10 nm gold (protein-A/10) and 15 nm (protein-A/15) were obtained from the Department of Cell Biology, University of Utrecht (The Netherlands), rabbit anti-goat gold-conjugated secondary antibodies from Jackson Immunoresearch laboratories (West Grove, Pennsylvania, USA), rabbit anti-goat and rabbit anti-mouse from Dako (Glostrup, Denmark).

Results

Detection of a single epitope

The combination of the primary and secondary antibodies will determine the result. Not only the primary antibody, which reacts with the antigen, but also the secondary gold conjugate determines labeling
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Table 4. Antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Clone</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth muscle actin</td>
<td>mouse</td>
<td>1A4</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Smooth muscle actin</td>
<td>mouse</td>
<td>HHF35</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Ulex europaeus agglutinin 1</td>
<td></td>
<td></td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>rabbit</td>
<td></td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>goat</td>
<td></td>
<td>SBA, Birmingham, AL, USA</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>goat</td>
<td></td>
<td>SBA, Birmingham, AL, USA</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>goat</td>
<td></td>
<td>SBA, Birmingham, AL, USA</td>
</tr>
<tr>
<td>Elastin</td>
<td>rabbit</td>
<td></td>
<td>Ron Wanders, Specicum Lab. Procreatie, AMC, Amsterdam, The Netherlands</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>rabbit</td>
<td></td>
<td>Dako, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

As an example, we will discuss labeling of actin, present in smooth muscle cells in aortic tissue, which can be achieved using a variety of techniques. When a monoclonal actin antibody (clone 1A4) in combination with protein-A/10 is used, the affinity of protein-A for the primary antibody is a limiting factor. The labeling efficiency for monoclonal mouse antibodies can be increased by introducing a "bridging antibody" (in this case a rabbit-anti-mouse antibody), since protein-A reacts strongly to rabbit antibodies. In this way, the labeling of smooth muscle cells in the aorta is increased. This is shown in Fig. 1A and 1B.

The same labeling efficiency can be achieved using a different primary antibody (clone HHF35) in combination with a bridging antibody (Fig. 1D). However, the combination of direct labeling of the anti-actin antibody (clone HHF35) and a secondary protein-A/10 conjugate shows almost no labeling activity (Fig. 1C). The choice of gold label is a second limiting factor. Adjacent tissue sections are incubated with the same primary antibody against actin and a secondary rabbit anti-goat gold-conjugated secondary antibody using different gold sizes of 6 nm and 12 nm. The labeling efficiency is increased using the smaller gold-sized secondary antibody. The results are shown in Fig. 2.

Single staining procedures are not only important to localize one specific antigen, but also to determine the sequence in the multiple labeling procedures. It is preferable to use the antibody which shows the least labeling as a first step in a double or triple labeling and to use a small gold conjugate to reduce steric hindrance.

Detection of two epitopes

Fig. 3 shows the results of a double-labeling experiment. Fig. 3A shows the labeling of a blood vessel in human heart tissue using two antibodies raised against vascular endothelial cells. *Ulex europaeus* agglutinin 1 (UEA-1), a membrane marker, and an antibody against the von Willebrand factor (vWf), staining the Weibel Palade bodies, were both used to stain endothelial cells. The staining protocol is as follows: UEA-1; rabbit anti-UEA-1 antibody; Protein-A/15; fixation with 1% glutaraldehyde in PBS; vWf primary antibody; protein-A/10, according to the protocol for double labeling (Table 3).

Fig. 3B shows a staining with primary antibodies against collagen type III and type VI in a human heart. The method used is a two-face method: both antibodies are goat anti-human. The second antibody is a rabbit anti-goat, followed by protein-A.

Fig. 3C shows a staining with anti-collagen type VI antibody and anti-elastin antibody in a human heart. The staining protocol is as follows: collagen type VI (rabbit anti-human); protein-A/10; fixation; anti-elastin antibody (rabbit anti-human).

Fig. 4 shows a double-staining of collagen type I and type III. Two-face immunolabeling was done to immunolocalize both types of collagen. The secondary antibodies are rabbit anti-goat. The 15 nm gold particles
Figure 4: Immuno-double labeling of collagen type I and type III in the extracellular matrix of a human heart with chronic congestive heart failure. The 15 nm gold particles represent collagen type I; the 30 nm gold particles represent collagen type III. The method is a two-face immunolabelling. Bar=0.5 µm.

represent collagen type I; the 30 nm gold particles represent collagen type III.

Detection of three epitopes

Fig. 5A shows a triple labeling using antibodies against elastin (rabbit anti-human), fibronectin and actin. The staining protocol is: anti-fibronectin antibody labeled with protein-A/10 nm and after fixation a mixture of the two remaining antibodies, using different-sized gold-conjugated antibodies. The fixation step between the first and second primary antibody is necessary to prevent cross reaction. The 5 nm gold particles represent actin; the 15 nm gold particles represent elastin.

Fig. 5B shows a staining with anti-collagen type VI antibody, anti-collagen type III antibody and anti-elastin antibody. A two-face method has to be used since all antibodies are polyclonal, with two from the same species (goat). The protocol is as follows: anti-elastin antibody; protein-A/5; fixation; anti-collagen type VI antibody; rabbit anti-goat; protein-A/15. The remaining anti-collagen type III antibody is applied at the other side of the grid and labeled with protein-A/10.

Discussion

The extracellular matrix is a relatively stable structure that surround cells and can act as a support. It is considered of great importance for the regulation of cell shape, cell migration, control of cell growth and differentiation (Hay, 1991). A diversity of extracellular matrix components exists, each possessing different functional characteristics: collagens are a source of strength to the tissue, elastin and proteoglycans are essential to matrix resiliency, and the structural glycoproteins help to create tissue cohesiveness. Together these components constitute an intricate network that is difficult to unravel by light microscopy. Electron microscopy can therefore greatly contribute to the study of the fine organisation of this matrix.

Unfortunately, extracellular matrix components are often poorly visible in routinely stained electron microscopic specimens. One method to improve the situation is a brief pre-staining of sections with diluted tannic acid general to increase the overall contrast of extracellular matrix components (Dingemans and van den Bergh Weerman, 1990). However, this method is not effective in the differentiation of elements that are morphologically identical (e.g., collagen types I and III), or nearly identical (e.g., fibronectin and elastin-associated microfilaments). The use of immunolabeling techniques, therefore, can be of importance for the study of extracellular matrix components. This is especially so when multiple labeling is applied for the simultaneous identification of several components in one specimen. As we have discussed, these methods can be of importance to reveal the subtle extracellular matrix changes that occur in some pathological processes.

Chronic CHF is characterized by an increase in extracellular matrix components (Heneghan et al., 1991; Schaper and Speiser, 1992; Yoshikane et al., 1992; Marijanowski et al., 1995). Cyanogen bromide analysis
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Figure 5: A. Immuno-triple labeling of elastin, fibronectin and actin in aortic tissue. The anti-fibronectin antibody is labeled with protein-A gold/10 nm (two arrowheads); anti-actin and anti-elastin antibodies are used in a mixture and labeled with 5 nm (one arrowhead) and 15 nm (three arrowheads) gold conjugated antibodies, respectively. B. Immuno-triple labeling of collagen type VI, collagen type III and elastin in the extracellular matrix of a human heart. A two-face method is used. The anti-elastin antibody is labeled with protein-A/5 nm (one arrowhead), anti-collagen type VI is labeled with a rabbit anti-goat antibody and subsequently protein-A/15 nm (three arrowheads). On the other side of the grid anti-collagen type III antibody labeled with protein-A/10 nm (two arrowheads) has been applied. Bar=0.5 µm.

and immunohistochemical analysis followed by microdensitophotometric quantification (Marijanowski et al., 1995) showed that the interstitial collagens are increased and the ratio between collagen type I and type III is changed in favor of collagen type I. The localization of both type I and type III collagen is established at the electron microscopic level using immunolabeling techniques. This technique provides more detailed information on the exact changes in hearts of patients with chronic CHF, since the endomysium and perimysium can be identified more easily at the ultrastructural than at the light microscopic level, especially since the
distribution into endomysium and perimysium is lost in hearts of patients with chronic heart failure.

Immu-no-electron microscopy clearly showed that the increase in endomysial collagens is a diffuse phenomenon, whereas that in the perimysium was much more inhomogeneous. Our studies also show an increase in type I collagen over type III, although these observations were not quantified (Marijanowski et al., 1995; see also Fig. 4).

We have shown that hearts of patients with chronic CHF not only display an increase in collagens but, in fact, present distinct alterations in the composition of the interstitial collagen network. It is likely that these structural changes provide the substrate for the progressive increase in myocardial stiffness. It thus appears that these further to understand the changes in the extracellular matrix components in chronic CHF.

References


Robinson TF, Cohen-Gould L, Factor SM (1983) Skeletal framework of mammalian heart muscle: ar-
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Rangement of inter- and pericellular connective tissue structures. Lab Invest 49: 482-488.


Discussion with Reviewers

K. Robinson: You assert that it is preferable in multiple labelling procedures to first apply the antibody which gives the lowest labelling index, and to use small gold probes to minimize steric hindrance from subsequently applied antibodies. Can such effects either entirely mask antigens, or give misleading results with respect to labelling intensity? If so, what steps could be taken to correct or at least adjust for this?

Authors: Yes, the possibility that antigens are masked partially, which may give misleading results cannot be overlooked. This effect can be avoided by the use a two-face method.

G. Pasquinelli: The authors state that when a multiple labelling is going to be performed, one should employ the smallest gold probe in the first step of the labelling procedure to avoid any steric hindrance at the next step; however, in the shown example (Fig. 3) it seems that the reverse is true (15 nm gold particles followed by the 10 nm ones).

Authors: This comment is basically correct. In the present example, however, it is known that the two antigens have different localizations (cell surface and Weibel-Palade bodies, respectively). Steric hindrance, therefore, seems unlikely in this particular case. Moreover, the two antibodies used in this case show a high and comparable degree of labeling, so that no problems caused by partial masking are to be expected.