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Immunoreactive Elastin in Benign Breast Tissues

An Immunoperoxidase Study

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Summary. Immunohistological localisation of elastin was achieved by means of the peroxidase-antiperoxidase method after preliminary trypsinisation of sections from 48 benign breast biopsies. The procedure allows retrospective examination of routinely formalin-fixed, paraffin-embedded breast tissue. In general the immunolocalisation of elastin showed a close microanatomical correlation with the fibres demonstrable in sections from the same blocks by standard elastic-fibre stains. Discrepancies between elastic-fibre stains and elastin immunoreactivity appear to relate to the enhanced avidity of the antibody for immature elastin. In this way sites of recent synthesis of elastin were demonstrated in the inner zone of the periductal elastica, sclerosing adenosis, and in the internal elastic lamina of breast arteries which displayed reduplication of the internal elastic lamina or intimal proliferation.

Key words: Elastin – Elastic fibres – Benign breast diseases – Artery – Immunohistochemical localisation

Elastin is the specific protein component of elastic fibres. Other elements found in the fibres, exemplified by collagen fibrils and glycoproteins of fibrillary and amorphous character, are less specific since they are also found in a wider range of connective tissues. Standard elastic-fibre stains currently do little to determine the relative proportions of these components in either normal or diseased elastic fibres.

Hitherto the hydrophobic nature of mature elastin has hindered immunohistochemical studies. Recently, antisera raised in sheep to immature elastin isolated from human foetal aortae (Barnard et al. 1982b) have potentially altered this situation. Application of the peroxidase-antiperoxidase method has demonstrated the feasibility of examining routinely formalin-fixed and paraffin-embedded human aortae (Barnard et al. 1982a) for immunoreactive elastin. Differential immunological reactivity of the antiserum

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with immature and mature elastin would be expected to provide differing intensities of immunostaining of immature and mature elastin in tissue sections. Accordingly, we wish to report the results of examination of a series of benign human breast lesions.

Materials and Methods

Breast Tissues

Tissue paraffin blocks were selected for study the diagnostic material received in the University Department of Pathology at the Bristol Royal Infirmary in the period 1979 to 1981.

The tissues were routinely fixed in fresh but unbuffered formol saline (10% formalin). All were serially sliced shortly after receipt before specimen radiographs were taken. Immersion in fixative ranged from 6 to 36 h before processing through graded alcohols and toluene with subsequent embedding in paraffin wax (melting point 56° C). Sections were dewaxed in xylene and rehydrated in graded alcohols at room temperature. All breast biopsies were routinely stained by haematoxylin and eosin, and subsequently by a modified (Davies 1973) Weigert's elastic – van Gieson, and by orcein. From the material were selected 48 cases: 12 near-normal breasts, 12 cystic mastopathies with little elastosis, 12 cystic mastopathies with focally pronounced elastosis, 12 cases of various benign breast lesions: periductal mastitis (6), fibroadenoma of breast (3), benign ductal papillomas (2), and traumatic fat necrosis (1). From one to four paraffin blocks were examined immunohistochemically from each of the 48 cases. Replication of examination of individual blocks was performed over the 18 months during which the material was assessed.

Additional paraffin sections, 5–7 μm thick, were cut from the blocks for the study. All sections were submitted to preliminary trypsinisation (0.1% Wellcome trypsin for 30 min at 37° C), and to treatment with 0.5% hydrogen peroxide in methanol to block endogenous peroxidase activity.

Raising of α-Elastin Antiserum

Elastin antigen was prepared as α-elastin peptides by hot oxalic acid extraction (Partridge et al. 1955) of insoluble elastin from pooled foetal human aortae. The amino acid content of the insoluble elastin resembled similar preparations of adult aortic elastin, and differed markedly from types I and III collagen and extracted aortic glycoproteins (Barnard et al. 1982b). The main difference between the foetal and adult elastin was the reduction in lysine and increase in desmosine and iso-desmosine in the latter. The antiserum was raised in sheep by a schedule of repeated nuchal inoculation with Freund's complete adjuvant (Difco). The sheep were bled one week after the last inoculation.

Antibody Characterisation

The poor immunogenicity of elastin precludes conventional agar gel immunoprecipitin reactions. Passive haemagglutination showed a reciprocal titre of 640 when the antielastin antiserum was exposed to tanned red cells coated with foetal human α-elastin peptides. The antiserum to foetal α-elastin peptides showed a four-fold preferential avidity for foetal as opposed to adult elastin, which gave a reciprocal titre of 128, in passive haemagglutination. No reaction at all was found in this system between the antiserum and aortic glycoprotein extracts, or with types I and III collagen. Similar results were found in competitive radioimmunoassay. Crossreaction with adult human elastin, but no competitive binding reaction with aortic glycoprotein or types I and III collagen was found (Barnard et al. 1982b).

Immunohistological Methods

After trypsinisation in 0.05 M tris/0.4% NaCl with 0.05% $CaCl_2$ at pH 7.8 the dewaxed paraffin sections were rinsed thoroughly with tris-saline buffer (pH 7.6), and then incubated for

10 min at room temperature in a moist chamber with 25% normal rabbit serum in tris-saline. The sections were next washed in tris-saline and incubated at room temperature with the α-elastin antiserum or normal sheep serum, both diluted 1 in 600 with tris-saline, for 45 min. After intermediate washes the sections were flooded with rabbit anti-sheep IgG (Nordic Laboratories) diluted 1 in 50. After further tris-saline washing the sections were then exposed at room temperature to sheep peroxidase-antiperoxidase complex (Cappel Laboratories) diluted 1 in 40. The exposures in the latter two steps were also for 45 min. The reaction product in the finally washed sections was developed with a solution of 2 mg ethylcarbazole and 0.5 ml, N,N dimethylformamide in 9.5 ml acetate buffer pH5 after the addition of 2 drops of H_2O_2 solution. After 5 min the sections were again rinsed, counterstained with haematoxylin and mounted in glycerin jelly under a glass coverslip.

Absorption of the α-elastin antiserum was performed by incubation at 4° C for 24 h. In order to achieve the working antiserum dilution of 1 in 600 0.5 ml of antielastin at a dilution of 1 in 300 was mixed with 0.5 ml tris-saline in which 1 mg of lyophilised α-elastin peptides were dissolved. Similarly, 0.5 ml of a solution containing 1 mg aortic glycoprotein extract (Barnard et al. 1982b) was also used to absorb 0.5 ml of antielastin diluted 1 in 300. After centrifugation the treated antisera were applied as in the method above.

Results

Immunostaining of the breast tissue sections was restricted to areas containing elastic fibres. The intensity of immunostaining varied with the dilution of the primary antiserum to foetal α-elastin. At dilutions of 1 in 50 and 100 a prozone effect with very weak staining was found. Maximum staining intensity was found at a dilution of 1 in 600, with diminishing reactivity at higher dilutions.

Omission of the primary elastin antiserum or of the anti-sheep immunoglobulin or peroxidase-antiperoxidase complex led to complete abolition of elastic fibre staining. No elastic-fibre staining was found in control sections using non-immune sheep serum instead of the anti-elastin. No staining of elastic fibres was obtained following exposure of sections to antisera directed against the unrelated antigens carcinoembryonic antigen, secretory piece, or epithelial membrane antigen. After absorption of the antiserum by α-elastin peptides there was complete loss of immunostaining of the periductal (Fig. 1), interlobular and vascular elastic fibres. The staining was unaffected by absorption of the antiserum by the glycoprotein extract.

Immunohistochemical Appearances of Tissues

Comparison was made between the elastic-van Gieson and orcein stained sections and the peroxidase-antiperoxidase preparations after application of the α-elastin antiserum. A systematic evaluation of the elastic fibres around the ducts, in the interlobular connective tissue and of the arteries and veins was made.

The maximum intensity of elastosis in the structures listed above was separately assessed in a representative block from each of the 48 cases. In most instances the assessment of periductal elastosis (Table 1) showed goood correlation between the standard elastic stains and the elastin peroxidase-antiperoxidase preparations (Fig. 2). With one exception the subjective

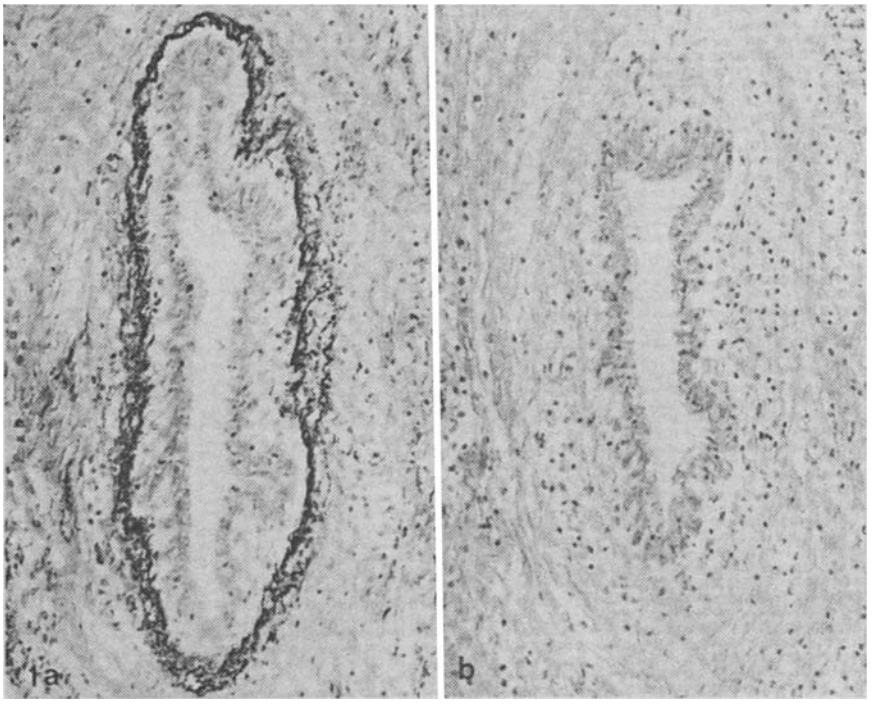

Fig. 1 a, b. Periductal elastica after peroxidase-antiperoxidase staining. **a** Antielastin antiserum shows discrete immunostaining of elastic fibres. **b** Preliminary absorption by α-elastin peptides completely eliminates elastic fibre immunostaining. Both ×150

Table 1. Comparison of assessments of periductal elastosis in elastic van Gieson stains and elastin immunoperoxidase preparations

Intensity elastin PAP reactivity	Density elastic staining		
	Weak	Moderate	Strong
Weak	27	1	1
Moderate	2	4	1
Strong	0	1	11

assessments were within one grade of one another. In general the interlobular connective-tissue elastic-fibres were uniformly stained in both elastic stains and the elastin-immunostained preparations. The distribution of interlobular elastic – sparse, moderate or dense – was similar in both types of preparation.

Obliterated and elastotic ducts in the vicinity of sclerosing adenosis tended to show weaker immunostaining (Fig. 3) than in the adjacent active

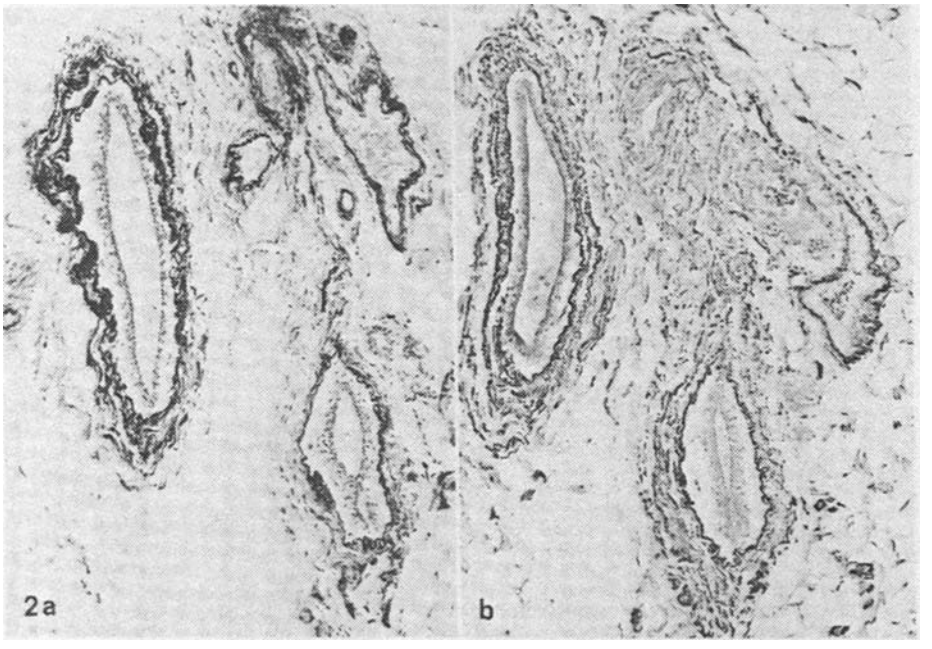

Fig. 2a, b. Three breast ducts (*left, right* and *lower*) together with two segments of a small artery (*upper centre*). **a** Orcein stains uniformly periductal elastica and arterial internal elastic lamina. **b** Peroxidase-antiperoxidase immunostaining of immature elastin. Periductal elastica well stained with accentuation of inner layer. Absent immunostaining of arterial internal elastic laminae. Both ×110

sclerosing adenosis. Hyperelastotic duct obliteration in periductal mastitis (Fig. 4) displayed equivalent staining reactions in the elastic stains and in the antielastin preparations. In elastic ducts which retained intact epithelial and myoepithelial layers, there was a slightly enhanced immunostaining of the internal layers (Fig. 2) of the periductal elastica as compared with standard elastic staining.

The vein wall and adventitial elastic fibres wer stained by both the elastic stains, and by elastin peroxidase-antiperoxidase procedure. Their intensity and microanatomical distributions were similar in all preparations. The internal elastic lamina of small and medium-sized muscular arteries showed a discrepancy in staining reactions. They were well stained by the standard elastic stains. However normal muscular breast arteries exhibited weak or inconstant immunostaining of the internal elastic lamina (Fig. 2). In contrast arteries which showed reduplication of the internal elastic lamina (Fig. 5), or intimal elastic-fibre proliferation were well stained by the anti-elastin.

Immunostaining with the elastin antiserum thus shows close correlation with the standard elastic fibre staining. Minor discrepancies were found in the reactivity of the internal elastic lamina of small arteries, the inner zone of the periductal elastica, and in active sclerosing adenosis.

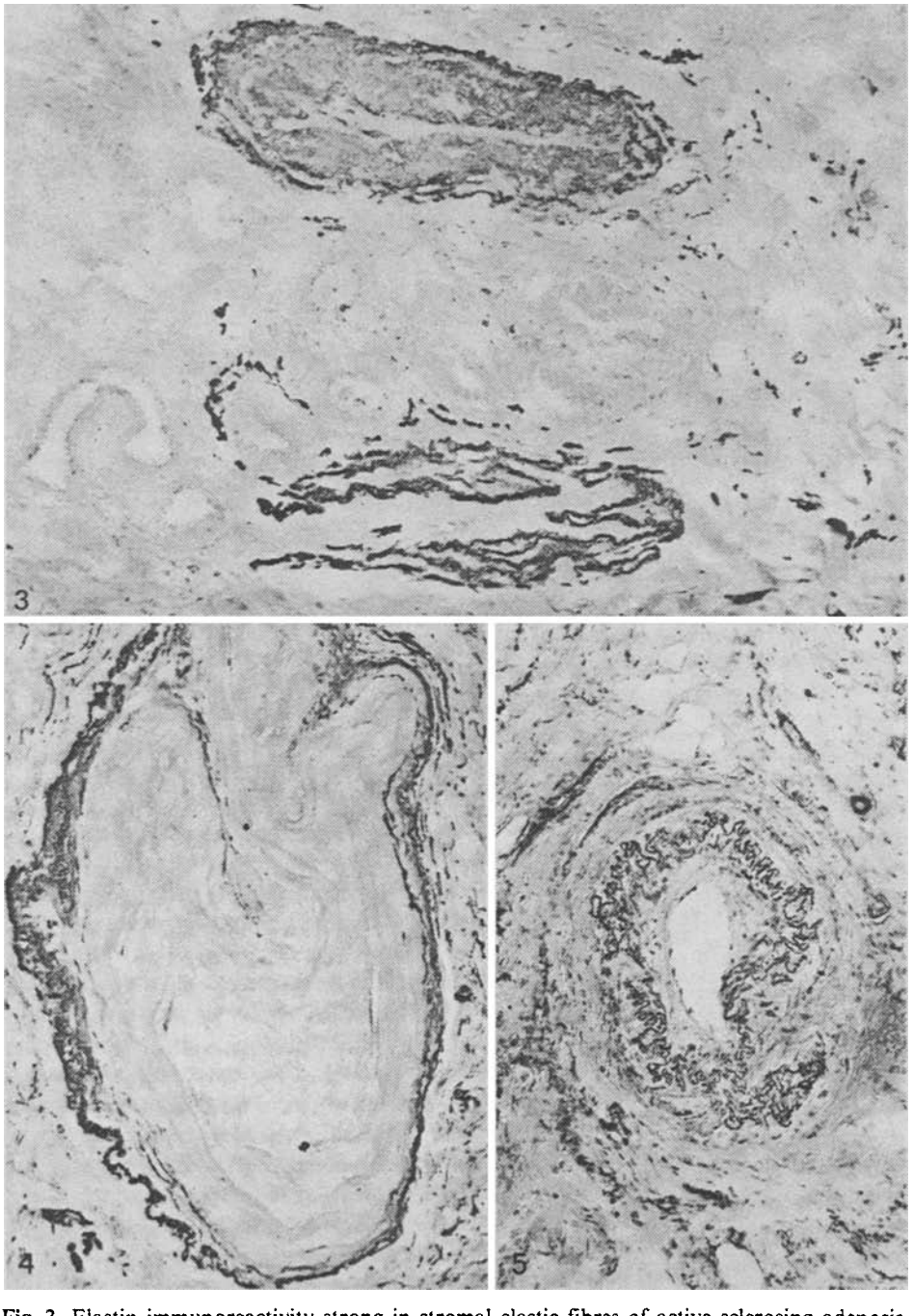

Fig. 3. Elastin immunoreactivity strong in stromal elastic fibres of active sclerosing adenosis (*lower half of field*). Weaker immunostaining of central elastic in obliterated and elastotic duct (*upper*). Antielastin immunoperoxidase × 150

Fig. 4. Elastic-fibre rim of obsolete obliterative periductal mastitis shows elastin immunoreactivity. Immunoperoxidase × 150

Fig. 5. Marked elastin immunostaining of reduplicated internal elastic laminae of breast artery. Immunoperoxidase × 120

Discussion

Elastin, when mature, is a crosslinked and hydrophobic protein which is poorly antigenic. While many antisera have been raised to elastin peptides from experimental animals (Daynes et al. 1977; Mecham and Lange 1980; Kucich et al. 1981) there has been limited success in developing antisera against human elastin. Darnule et al. (1980) raised antibodies to human lung elastin peptides which demonstrated some cross-reactivity with human aortic elastin, and Kucich et al. (1981) have obtained antibodies to human lung and aortic elastin peptides. However these antibodies have not been used for immunolocalisation studies. Our starting material was immature, only partly crosslinked elastin obtained from human foetal aortae. The relatively poor performance of rabbits in developing antisera to elastin led us to use sheep, which have the additional advantage of yielding large volumes of antisera at a given stage in the immunisation schedule. The cross reaction found in passive haemagglutination and competitive radioimmunoassay indicate that the sheep antibody has a preference for immature, or relatively recently synthesised elastin. This differential avidity seems likely to underlie the exceptional differences found in this study between standard elastic-fibre staining and the immunoreactivity in the breast lesions.

Earlier work has shown that our antibody is capable of immunolocalisation in human tissue using an indirect immunofluorescent method (McCullagh et al. 1980). The disadvantage of immunofluorescence in studying an intrinsically autofluorescent substance such as elastin are obvious. More recently we have shown that the peroxidase-antiperoxidase method is feasible for the study of elastin in human adult aortae (Barnard et al. 1982a). In this present study we have extended the method to the examination of breast tissue.

Elastin fibres are composed of several distinct constituents, including collagen, various glycoproteins and the matrix in addition to the elastomeric protein elastin. The proportions of these elements appear to vary in both normal and pathological elastic fibres (Kadar 1980). Unfortunately neither the traditional empirical elastic fibre stains nor even electron microscopy are capable, at present, of reliably detecting variations in these constituents. The structured and focal nature of elastotic deposits are barriers to meaningful biochemical analysis of tissue homogenates; equally stereological methods require to take into account the topological context of elastotic lesions (Davies and Barnard 1982).

The general correspondence between the elastic stains and the elastin immunolocalisation supports the apparent immunospecificity of the antiserum. This correspondence was seem in most elastic deposits associated with breast parenchyma, the interlobular connective tissue and in adventitial vascular elastic fibres. The two main exceptions to this correlation deserve comment. We feel that the preference of our antibody for newly synthesised elastin explains the anomalies and contributes a method of studying elastin metabolism in fixed tissues.

The intensified immunostaining of active sclerosing adenosis and the inner layers of the periductal elastica contrasted with the uniform elastic staining of these fibres. Many types of cells are thought to be capable of cellular synthesis of elastin. The association of ductal elastosis in benign breast lesions, notably sclerosing adenosis (Davies and Haines 1977), a lesion which displays myoepithelial cell proliferation, suggests that myoepithelium may be involved in elastin synthesis. The enhanced staining of the inner layer of the periductal elastica, closest to myoepithelium, would also be expected.

Our findings in regard to the internal elastic lamina of the muscular breast arteries are also of interest. Earlier immunohistological investigations have shown good staining of the internal elastic lamina by the same antiserum in the aorta in the indirect immunofluorescent (McCullagh et al. 1980) and immunoperoxidase (Barnard et al. 1982a) systems. The normal smaller tributary arteries in the breast have given consistently different reactions. The internal elastic lamina of these arteries displays relatively weak or absent immunoreactivity despite uniform elastic staining. This situation is dramatically altered in the presence of reduplication of the internal elastic lamina or of less orderly intimal fibroelastosis. In both circumstances there is a marked increase of immunoreactivity of the internal elastic lamina which probably reflects recent elastin synthesis.

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