HISTOLOGICAL GRADING, HISTOCHEMISTRY, AND ELECTRON MICROSCOPY
OF SCIRRHOUS CARCINOMA OF THE BREAST

Rikuo Machinami*
Chester Beatty Research Institute, Institute of Cancer Research, and Royal Cancer Hospital, University of London

 Twenty-six scirrhous carcinomas of the breast were divided into three histological grades of malignancy; low (Grade I), intermediate (Grade II), and high (Grade III), and the correlation of the grades with histochemical and electron microscopic findings in both tumor cells and host tissues was examined. The tumor cells contained increased amounts of lysosomal acid phosphatase and \( \beta \)-glucuronidase. This increase was most marked in Grade I and II tumors and the increase was consistent with lysosome-like fine structures. Both intracytoplasmic lumina and microvilli against stroma were characteristic findings of carcinoma cells and they were mostly found in Grade I and II tumors. Segments of intact basal laminae and myoepithelial cells were also found in Grade I and II carcinomas.

 The stroma contained moderately increased amounts of intracellular acid phosphatase and \( \beta \)-glucuronidase, independent of tumor grade. The stroma also contained a large amount of acid mucopolysaccharide ground substance, irrespective of the three grades. There was a striking difference in the ultrastructural organization of the stroma between normal and neoplastic tissues. Although fragmented elastic fibers and increased amount of acid mucopolysaccharide granules, and macrophages rich in phagolysosomes were prominent fine structures of the stroma of carcinomas, there was no apparent difference in them among the three grades of malignancy.

 It is a pathologist's earnest desire to predict the prognosis of carcinoma from histopathology of the tumor; one of the earliest such attempts was Broders' grading for squamous cell carcinoma. Similar histological grading for breast carcinoma has been introduced by Patey and Scarff, later modified by Bloom and Richardson. Although the biological behavior of carcinoma is the result of interaction between tumor cells and host tissues, only the epithelial elements of breast carcinoma were used for these histological grading systems and good correlation with prognosis has been obtained.

 Increase in stainable lysosomal enzymes in tumor cells has been reported on several human carcinomas. This seems to correspond closely to some biochemical studies indicating that lysosomal enzymes released from tumor cells facilitate invasion. One might well expect that the degree of potential malignancy would be parallel to the activity of lysosomal enzymes in tumor cells.

 The aim of this study is to see if there is any correlation between the histological grading and the histochemical and ultrastructural findings both in tumor cells and stroma, and to evaluate which factors are responsible for

* Present address: Department of Pathology, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113 (町並隆生).
invasive growth of scirrhous breast carcinomas.

MATERIALS AND METHODS

Twenty-six scirrhous carcinomas were studied in fresh mastectomy specimens obtained from the Royal Marsden Hospital, London. Several blocks of tissue measuring about 2 cm³ were removed from the edges of each tumor and processed for routine histology, enzyme histochemistry, and electron microscopy. Uninvolved breast tissues from 13 patients with fibrocystic disease or fibroadenomas were used as controls. The ages of patients with scirrhous carcinoma ranged from 28 to 77 years (average 54.5) and those of the non-neoplastic group varied between 25 and 70 years (average 40).

For light microscopy, tissues were fixed in Bouin's fluid and 4–5 μm paraffin sections were stained with Hematoxylin and Eosin, periodic acid-Schiff and Alcian Blue (PAS-AB), and Elastin-Van Gieson.

The carcinomas were placed in one of the three grades of malignancy; low (Grade I, Photo 1), intermediate (Grade II, Photo 2), or high (Grade III, Photo 3) according to the method of Bloom and Richardson, which is based on the three histological factors: (a) degree of structural differentiation as shown by the presence of tubular arrangement of the cells, (b) variation in size, shape, and staining of the nuclei, and (c) frequency of hyperchromatic and mitotic figures.

Histochemistry for acid phosphatase was carried out according to the method of Burstone modified by Barka, and for β-glucuronidase by the method of Hayashi; the tissues were fixed in formal calcium for 24 hr and stored in gum sucrose for 1 to 5 days. Frozen sections were cut at 6 μm and incubated with the appropriate substrate for 30 min at 37°C.

For electron microscopy, 8 to 12 blocks measuring about 1 mm³ were fixed for 2 hr in formaldehyde-glutaraldehyde solution. They were then post-fixed in 1% OsO₄ for 30 min and embedded in Araldite. Ultrathin sections were stained with an alkaline lead solution and examined in a Philips EM 300 microscope.

RESULTS

General Histology The 26 scirrhous carcinomas, graded according to the criteria of Bloom and Richardson, showed the following distribution: Grade I, 7 cases; Grade II, 10 cases; Grade III, 9 cases. About one-half of the tumors were lightly infiltrated with macrophages, lymphocytes, or plasma cells. The amount of collagen fibers present was variable, both between tumors and in different parts of the same tumor. In general, the most fibrous lesions contained the smallest number of tumor cells. The number of fragmented and thickened elastic fibers was increased in 15 of 26 scirrhous carcinomas but no correlation with grade was established.

The 13 specimens of uninvolved breast tissue from patients with fibrocystic disease or fibroadenoma showed no unusual features. Abundant collagen fibers were seen in the interlobular zones. A fine laminar arrangement of intact elastic fibers was sometimes noted around the larger ducts; fragmented elastic fibers were not seen.

Histochemistry

Periodic Acid-Schiff (PAS) and Alcian Blue Reactions: Tumor cells in 16 of the 26 scirrhous carcinomas (5 from Grade I, 8 from Grade II, and 3 from Grade III) showed positive cytoplasmic staining with PAS and Alcian Blue, the reaction product forming sharply localized deposits in all but three instances. Grade I and II carcinomas, showing tubule formation, often contained free PAS-AB positive material in their lumina. The most striking feature was the intense Alcian Blue staining of the stroma, found in 24 of the 26 carcinomas. PAS-positive material in the stroma was not seen.

The control breasts, the average age of which was 15 years younger than that of the carcinomas, showed somewhat different pattern of PAS-AB staining. Some PAS-AB positive material was observed free in the ducts though the ductal lining cells were consistently negative. Most strikingly, the connective tissue stroma showed little or no staining with PAS or Alcian Blue.

Acid Phosphatase and β-Glucuronidase Reactions: Abundant acid phosphatase was demonstrated in the tumor cells of 15 of the 26 scirrhous carcinomas; stainable β-glucuronidase was also increased in 11 of these
The stainable enzymes tended to be most prominent in the Grade-I neoplasms, the reaction products being located predominantly at the luminal surface (Photo 4). More discrepant results emerged from the less-differentiated tumors of Grades II and III; less stainable material was demonstrable, and acid phosphatase and β-glucuronidase tended to be unequally distributed; most commonly there was a predominance of stainable acid phosphatase. Increased amount of stainable acid phosphatase and β-glucuronidase was also demonstrated in the stroma of most scirrhous carcinomas (22 of 26). In contrast to the staining pattern noted for these enzymes in the tumor cells, the stromal staining bore no relation to grade. Acid phosphatase and β-glucuronidase were usually increased synchronously in the stroma but discrepancies were sometimes encountered, the commonest being predominance of stainable acid phosphatase. The reaction products for both enzymes were localized to fibroblasts and also macrophages; extracellular staining was never seen.

In normal breast tissue, the mammary epithelium contained small amounts of stainable acid phosphatase and β-glucuronidase. Usually present in approximately equal amounts, the reaction products formed discrete granules which were often concentrated towards the luminal surface or situated at the base of ductules. Similarly, small and roughly equal amounts of these two enzymes were demonstrable in the normal stroma.

Main histochemical findings in scirrhous breast carcinomas are summarized as follows: The tumor cells contain increased amounts of lysosomal acid phosphatase and β-glucuronidase. This increase is most marked in the Grade I and II tumors. The stroma contains moderately increased amounts of intracellular acid phosphatase and β-glucuronidase, independent of tumor grade. The stroma also contains large amounts of acid mucopolysaccharide ground substance stained with Alcian Blue irrespective of tumor grade.

Electron Microscopy
General Observations: An attempt was made to link ultrastructural features of tumor cells with the corresponding characteristics used for the grading in the light microscope. Well-formed tubular structures were seen in 12 out of the 26 scirrhous carcinomas in the electron microscope. Ten of them were in tumors graded as I or II and showed tubule formation in the light microscope; there was a correlation between the light and electron microscopy. Variations in nuclear structure corresponding to the pleomorphism and hyperchromatism used in conventional grading were not sufficiently consistent in the electron microscope to distinguish the three grades of carcinoma.

Intracytoplasmic Vacuoles Lined by Microvilli (Photo 6): They were found in 13 of the 26 carcinomas, 10 of them in Grade I and II neoplasms. Similar but rather smaller structures were seen in a few acinar cells in 3 out of 13 normal breasts.

Lysosomes and Endoplasmic Reticulum: Lysosomes were identified in 24 of the 26 carcinomas examined. The exceptions were two Grade-III tumors which contained only traces of stainable lysosomal enzymes in the light microscope. Lysosomes were largest and most numerous in the 7 Grade-I carcinomas (Photo 6) and correlation with previous histochemical findings was close. However, there were carcinomas with increased amounts of stainable enzymes which did not contain prominent lysosomes when examined in the electron microscope; this will be discussed later.

Parallel lamellae of endoplasmic reticulum were found in a few tumor cells from 11 carcinomas; 5 of them were Grade III neoplasms. Correlation with the distribution of stainable β-glucuronidase in the light microscope was not close.

Small lysosomes were seen in most of the normal mammary epithelial cells though they varied in number. They were often concentrated near the lumina or at the base of
the ducts or ductules, thus corresponding rather closely to the distribution of stainable lysosomal enzymes previously observed in the light microscope.

Cell-to-Cell Contact: Most epithelial cells in both normal and neoplastic tissue lay in close contact with each other (Photo 6). Apparent loosening of cell-to-cell contacts was seen in 6 carcinomas but similar changes were also noted in 4 of the control tissues. Desmosomes were identified in all the scirrhouous carcinomas examined. No differences were found in their number and configuration in neoplastic and normal epithelium, and no variations were detected among carcinomas of different grades.

Basal Laminae: Epithelial cells in normal breast tissue were always surrounded by basal laminae. Segments of basal laminae were identified in 12 of the 26 scirrhouous carcinomas and were related to grade. They were present in 5 out of 7 Grade-I tumors and 6 out of 10 Grade-II tumors, but only in 2 out of 9 Grade-III lesions. Most of the basal laminae were morphologically similar to the laminae from control breast tissues but their relation to the plasma membrane was altered. In normal breast tissue there was a clear gap between the plasma membrane and basal laminae; in scirrhouous carcinomas this gap was often obliterated (Photo 5). Microvilli protruding from tumor cell membranes were sometimes observed in direct contact with the surrounding ground substance (Photo 7). This feature, found in 2 from Grade I and 3 from Grade II, was never encountered in the control tissues.

Myoepithelial Cells: These elements, regularly found in normal breast tissues, were observed in 8 carcinomas. They were encountered most often in Grade I and II neoplasms; 7 out of 17 Grade-I and -II tumors but only one in Grade III carcinoma. Usually situated at the periphery of the lesions, they closely resembled myoepithelial cells from normal breast tissue (Photo 8) except for their somewhat elongated appearance and occasional degenerative changes. Most of the myoepithelial cells observed in the carcinomas were accompanied by intact basal laminae.

Stroma: Normal epithelial cells, uniformly covered by basal laminae, were surrounded by collagen fibers of varying thickness; acid mucopolysaccharide granules occurred outside these collagen layers. Cells in scirrhouous carcinomas were in some parts directly surrounded by collagen (Photo 10) but, in regions where the basal laminae had been lost, tumor cells abutted directly on to ground substance (Photo 9). No constant ultrastructural differences were found in collagen from normal breast tissue and scirrhouous carcinomas apart from equivocal observations in two Grade-III tumors where the collagen fibers were abnormally fine and attenuated. There was no morphological evidence to suggest that direct contact between tumor cells and collagen showing degradation such as loss of banding pattern or becoming amorphous material. Fragmented elastic fibers lying in a matrix of acid mucopolysaccharide were seen in 8 of the carcinomas (Photo 11) but in none of the controls.

Most specimens of normal breast tissue contained few or no acid mucopolysaccharide granules. Acid mucopolysaccharide ground substance was, however, a prominent feature in 23 of the 26 carcinomas examined; in many of them it was seen in direct contact with tumor cells.

Main ultrastructural findings in scirrhouous breast carcinomas are summarized as follows: Grade I and II tumors show some or all of the following findings; intracytoplasmic vacuoles, prominent lysosomes, segments of intact basal laminae, and myoepithelial cells. No abnormalities were seen in cell-to-cell contact and desmosomes. The stroma contains abundant acid mucopolysaccharide granules. Fibroblasts and collagen fibers show no consistent abnormality. There is fragmentation of elastic fibers.
Discussion

Bloom and Richardson5) have divided breast cancer into three grades of malignancy depending upon histological criteria and a good correlation with prognosis has been obtained. This can suggest that the three histological grades might well correspond to biological behavior of breast cancer; i.e., Grade III carcinomas would be more invasive than Grade I carcinomas. Invasion is one of the main characteristics of malignancy. It can be considered as the result of mutual interaction between neoplastic cells and host connective tissues,14) and the factors in both tumor cells and stroma which might be responsible for invasion should be discussed separately.

Tumor Cells, Basal Laminae, and Myoepithelial Cells A few different findings from normal epithelial cells have been obtained in several carcinomas in this investigation. Most of them appear to be correlated with differentiation rather than invasive property of tumor cells.

The intracytoplasmic vacuoles lined by microvilli have been called by various names such as intracytoplasmic or intracellular lumina, or intracytoplasmic ductules or ducts; they have been described as one of the characteristic features of breast cancer.3, 11, 19, 25, 31, 34) Although these structures have also been found in normal breasts1, 19) and they cannot be pathognomonic to breast cancer cells, increased number of these luminae was one of the prominent differences from normal breast. Since these structures were more often found in Grade I than Grades II and III, they might be related to differentiation of carcinoma cells. Localized PAS- and Alcian Blue-positive material was only seen in carcinoma cells and was closely related to intracytoplasmic lumina, which suggests that PAS and Alcian Blue stainings are useful for detection of the lumina as well as carcinoma cells.31)

Following the development of the lysosome concept,10) there have been several reports that lysosomal enzymes released from tumor cells facilitate invasion.27, 32) Increase in stainable lysosomal enzymes in tumor cell that has been reported on several human carcinomas was found in more than half of our scirrhous breast carcinomas. This does not necessarily support the previous results, because both the increase in stainable lysosomal enzymes and the lysosome-like ultrastructures were more often seen in Grade I and II carcinomas; i.e., both of them represent differentiation of tumor cells rather than their invasiveness.

Most of the lysosome-like structures were similar to the secretory granules which had been described by several authors.3, 11, 31, 34, 36) Although neither primary nor secondary lysosomes can be recognized solely from their fine structure8) and some of the secretory granules are included in lysosomes,20) the presence of the stainable lysosomal enzyme-positive material in the tubules of the scirrhous breast carcinomas appears to suggest that most of these structures are secretory granules.

There were discrepancies between stainable acid phosphatase and β-glucuronidase as well as the two stainable lysosomal enzymes and lysosome-like fine structures. This seems consistent with dual localization of β-glucuronidase in lysosomes and endoplasmic reticulum,13) but there was no correlation between the stainable β-glucuronidase and the parallel lamellae of endoplasmic reticulum. The stainable acid phosphatase and lysosome-like ultrastructure were also discordant which might suggest free acid phosphatase in the cytoplasm of breast carcinoma cells.6, 22)

The relationship between the process of neoplastic invasion and the presence or absence of basal laminae has been discussed by several authors.24) In the present study the absence of basal laminae was parallel to grades and it can be an indication of malignancy, but the mechanism of its loss is obscure. Two kinds of basal laminae were
distinguishable. The one was often present at the base of myoepithelial cells with apparent clear zone (lamina lucida). This type might be the remaining basal laminae of normal ducts or ductules. The other type was with direct contact to tumor cells without lamina lucida, which appears to be newly formed basal lamina through interaction between tumor cells and ground substance.35)

The microvilli in direct contact with ground substance, which were peculiar to infiltrating breast carcinoma cells, have been described by few authors.35) These structures were only seen in Grades I and II; this might suggest that the microvilli do not indicate invasive property, such as ameboid motility, but differentiation of carcinoma cells.

The absence of myoepithelial cells in the electron micrograph seems to be correlated with degree of malignancy. There was no apparent deviation of the fine structure of myoepithelial cells from normal ones except elongated appearance; this might suggest that they are host myoepithelial cells left behind at the periphery of carcinoma cells29) under the mechanical pressure of tumor cells.37) Since intracytoplasmic lumina was present in carcinoma cells rich in cytoplasmic filaments in this investigation, these filaments seem to be tonofilaments.11,33,34,35) There was no evidence that scirrhou5s carcinomas originate from myoepithelial cells.22)

**Stroma** The difference between normal and neoplastic breasts was prominent in the stroma. There was a striking difference in the ultrastructural organization of the stroma between normal and neoplastic conditions. As described elsewhere,24) a layer of collagen fibers with various thickness could be seen in normal breasts beneath the basal laminae, whereas in carcinomas this layer was broken and tumor cells were in direct contact with mucopolysaccharide and collagen fibers. Epithelial–stromal junction, a hypothetical functional unit in the transport of materials to and from the epithelium,24) seems to be destroyed in carcinomas.

No apparent change could be found in most of the collagen fibers except fine fibrillar fibers in two carcinomas; this may suggest that active damage of collagen fibers by tumor cells is not likely.

Hyperelastosis of the mammary ducts in scirrhou5s carcinomas of the breast has been described by many authors since more than 100 years ago.9) Fragmented and thickened elastic fibers were increased in amount in our breast carcinomas, but its relation to grade was not clear.20) Tiny fragmented elastic fibers were found in direct contact with tumor cells in the electron microscope. This does not necessarily suggest the direct effect of carcinoma cells on elastic fibers, because similar fragmented fibers were present in the stroma a little distant from tumor cells.

Increased amount of acid mucopolysaccharide in the stroma of breast carcinoma has been found with PAS-AB staining.23) The finely granular ultrastructure of the stroma is similar to the fine structure of the cartilage matrix.21) The cartilage has none of its own blood vessels and nutritive requirements of chondrocytes are presumably being supplied only by way of diffusion through ground substance.4) This suggests that the increase in Alcian Blue-positive material in the stroma which might be produced by fibroblasts is useful for tumor cells in absorbing nutrient from the host.

The increase in stainable lysosomal enzymes in the stroma, mainly in macrophages, was a constant feature of the stroma of scirrhou5s breast carcinomas. This finding is not consistent with previous reports that the macrophage contributions are only part of the increase in lysosomal enzymes in tumor tissue.

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EXPLANATION OF PLATES

Photo 1. A Grade I carcinoma showing marked tubule formation. H-E. ×400.

Photo 2. A Grade II carcinoma showing slight tubule formation. H-E. ×400.

Photo 3. A Grade III carcinoma showing no tubule formation. H-E. ×400.

Photo 4. Stainable acid phosphatase in a Grade I carcinoma. Cells show granular reaction products which are sometimes concentrated at the luminal surface. There is little staining in the surrounding collagen fibers. Acid phosphatase staining. ×920.

Photo 5. A tumor cell with basement membrane found in a Grade II carcinoma. No clear zone (lamina lucida) can be seen between them. Compare with Photo 8. Glycogen particles (Gl) are prominent in the tumor cell. ×24,000.

Photo 6. Zone containing tubules in a Grade I carcinoma. Numerous lysosome-like structures (arrow) and several intracytoplasmic vacuoles lined by microvilli (V) can be seen around a lumen (L). D: Desmosomes. ×9,600.

Photo 7. A tumor cell from a Grade II carcinoma showing microvilli (arrow) in direct contact with the surrounding stroma. C: Collagen fibers. ×24,000.
Photo 8. A myoepithelial cell (MY) on the edge of a Grade I carcinoma. Closely similar to a myoepithelial cell from normal breast tissue, it contains abundant cytoplasmic filaments and aggregated mitochondria. A clear zone (arrow), the lamina lucida, is seen between the myoepithelial cell and basement membrane. ×24,000.

Photo 9. Cells from a Grade I carcinoma directly surrounded by collagen fibers (C) or granular material thought to be acid mucopolysaccharide (Am). ×9,600.

Photo 10. A tumor cell from a Grade III carcinoma in a collagenous stroma. There is no apparent degeneration of collagen fibers. Arrow head: Lysosome-like structure. ×24,000.

Photo 11. Fragmented elastic fibers (arrow head) can be seen close to tumor cells (T) and macrophages (M), the latter containing phagolysosomes. F: Fibroblast, C: Collagen fibers. ×9,600.

H-E = Hematoxylin-Eosin staining