Virchows Arch [Pathol Anat] (1981) 393:193–203

Virchows Archiv A

Ultrastructural Observations on Cell Death by Apoptosis in the "Resting" Human Breast

D.J.P. Ferguson* and T.J. Anderson

Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh, Scotland

Summary. For the first time the process of epithelial cell deletion was studied within the parenchymal component of the "resting" human breast. The dying cells were initially recognised by specific nuclear changes involving peripheral condensation of the chromatin and nucleolar disintegration. At this stage the cells were retracted from the lumen and had lost desmosomal connections with their neighbours. Within the cytoplasm, there was evidence of ribosomal detachment from the endoplasmic reticulum with the formation of ribosome aggregates. The majority of dying cells were phagocytosed at this stage although a few underwent further morphological changes. These involved blebbing and fragmentation of the nucleus followed by cytoplasmic fragmentation. The dying cells and cell fragments were phagocytosed by epithelial or myoepithelial cells as well as mononuclear phagocytes and undergo lysosomal digestion within the phagosomes. These progressive morphological changes were consistent with cell deletion occurring by apoptosis.

Key words: Cell death – Epithelium – Breast – Human – Apoptosis

Introduction

There have been a number of studies on the ultrastructural appearance of the parenchymal component (ducts and lobules) of the "resting" human breast but the process of cell deletion was not examined (Tannenbaum et al. 1969; Ozzello 1971; Stirling and Chandler 1976, 1977). The characterisation of cell death in healthy adult tissue has received little attention in comparison to that caused by toxins or ischaemia. In the last decade it has been shown that there are two distinct mechanisms of cell death. The first is the long established process of "coagulative" necrosis which normally involves groups of cells affected by adverse conditions (Trump and Mergner 1974). The second mechanism has been termed apoptosis; it affects individual cells and has been shown in

Offprint requests to: Dr. T.J. Anderson

* Present Address: Electron Microscopy Unit, Marischal College, University of Aberdeen, Aberdeen, Scotland

0340-1227/81/0393/0193/$02.20

certain cases to be under physiological control (Kerr et al. 1972; Wyllie et al. 1980). To date the most efficient way of differentiating between cells undergoing necrosis and apoptosis is based on the identification of specific morphological changes seen at the ultrastructural level (reviewed Wyllie et al. 1980). In this paper we describe the ultrastructural features of cell death by apoptosis within the lobules of the "resting" human breast.

Materials and Methods

Samples of normal breast tissue were obtained from biopsies of women of reproductive age (17–40). The criteria of normality was the same as that described previously (Ferguson and Anderson 1981). The biopsies were obtained directly from the operating theatre and 1 mm cubes of tissue were quickly excised and placed in 3% glutaraldehyde in cacodylate buffer pH 7.2. The blocks were fixed overnight, washed in cacodylate buffer, and post fixed for 1 h in 1% osmium tetroxide in cacodylate buffer. Dehydration was performed through an ethanol series followed by propylene oxide treatment after which the tissue was embedded in Araldite. Sections, 1 μm thick, were stained with toluidine blue and examined with the light microscope to identify ductules with cells which appeared to be undergoing apoptosis. Thin sections, obtained from suitable areas, were stained with uranyl acetate and lead citrate prior to examination with an AEI Corinth 275 electron microscope.

The ultrastructural localisation of acid phosphatase activity was based on the Gomori technique and was carried out using the incubation medium described by Barka and Anderson (1962) along with suitable controls.

Results

The parenchyma of the human breast consists of 12–20 branching duct systems which radiate from the nipple. At the ends of the terminal ducts are the lobules, each of which comprises a number of blind ending ductules which connect with the intralobular portion of a terminal duct. The ducts and ductules are basically similar in structure having a continuous layer of epithelial cells with an underlying discontinuous layer of myoepithelial cells (Fig. 1). In addition a few cells of the monocyte/macrophage system are observed between the epithelial and myoepithelial layers (Fig. 1).

Cell death by apoptosis was observed to occur in a few scattered cells within both ducts and ductules. The earliest morphological change which could be used to identify cells undergoing apoptosis is seen in the nucleus. This change was characterised by condensation of the chromatin into aggregates of various sizes which abut on the nuclear membrane (Fig. 2). This is in comparison to the nuclei of the healthy cells which have a homogeneous appearance (Fig. 2). At this stage the apoptotic epithelial cell was retracted from its luminal position and was normally observed between the epithelial and myoepithelial layers (Fig. 2). We found no evidence for apoptotic cells being sloughed off into the lumen. At this early stage of apoptosis the dying cell had lost desmosomal connections with its neighbours. However, there were no obvious changes in the cytoplasmic organelles including the mitochondria, although a few membrane bound vacuoles with osmiophilic contents were observed (Fig. 2).

In cells presumed to be at a later stage in the process, the margination of the chromatin forms crescentic caps at the periphery of the nucleus (Fig. 3).

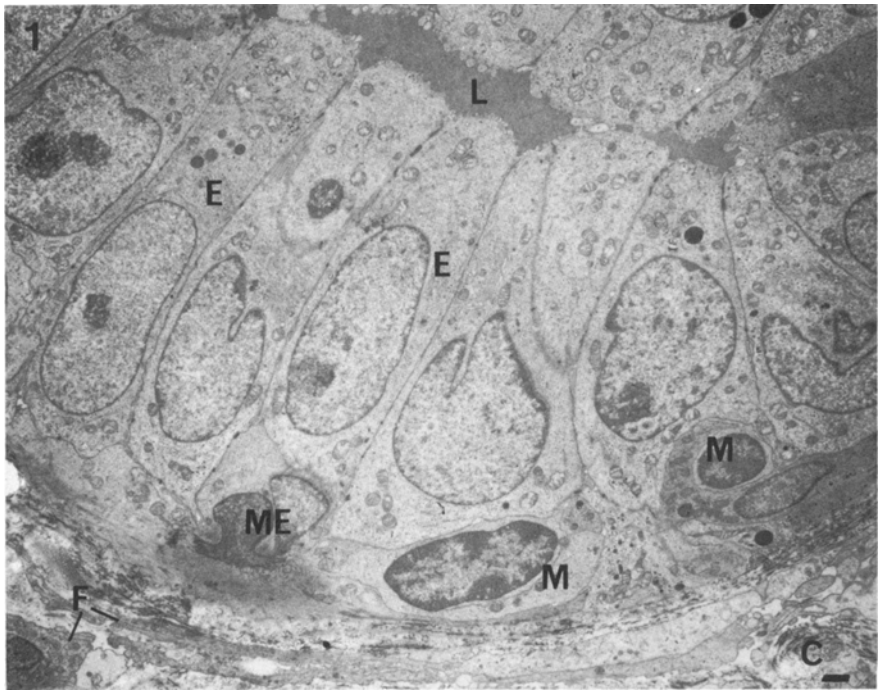

Figure 1 represents the general appearance of the parenchymal component of the "resting" human breast with **Figures 2–13** illustrating various stages in the process of apoptosis observed within the epithelium. A single bar (−) on a micrograph represents 1 μm and a double bar (=) 100 nm. The following abbreviations are used throughout: *C*, connective tissue; *CH*, condensed chromatin; *E*, epithelial cell; *ER*, endoplasmic reticulum; *F*, fibroblast; *L*, lumen; *M*, mononuclear phagocyte; *ME*, myoepithelial cell; *MI*, mitochondrion; *N*, nucleus; *NM*, nuclear membrane; *NP*, nuclear pore; *NU*, nucleolus; *R*, ribosome; *RA*, ribosome aggregate; *V*, vacuole with osmiophilic contents

Fig. 1. Part of a section through a ductule showing the layer of epithelial cells with underlying myoepithelial cells. Two mononuclear phagocytes are situated between the myo- and epithelial layers. × 3,600

The remnants of the nucleolus consisted of loose granular material (Fig. 3). In certain cases the nucleus also contained a granular structure comprised of 25 nm particles which probably represents aggregation of the granular component of the nucleolus (Figs. 3 and 4). Within the cytoplasm of a number of dying cells there was evidence of ribosome detachment from the membranes of the endoplasmic reticulum (Fig. 6) with the formation of ribosome aggregates of various sizes (Figs. 4 and 6). There was a marked similarity between the nuclear granular aggregates and the ribosome aggregates (Fig. 4).

At this stage many of the apoptotic cells were apparently phagocytosed by neighbouring epithelial (Fig. 9) or myoepithelial cells or mononuclear phagocytes. However, some of the apoptotic cells underwent further structural changes while still located intercellularly. The nuclear membrane becomes convoluted and blebbing of the nuclear surface at the sites of chromatin condensation

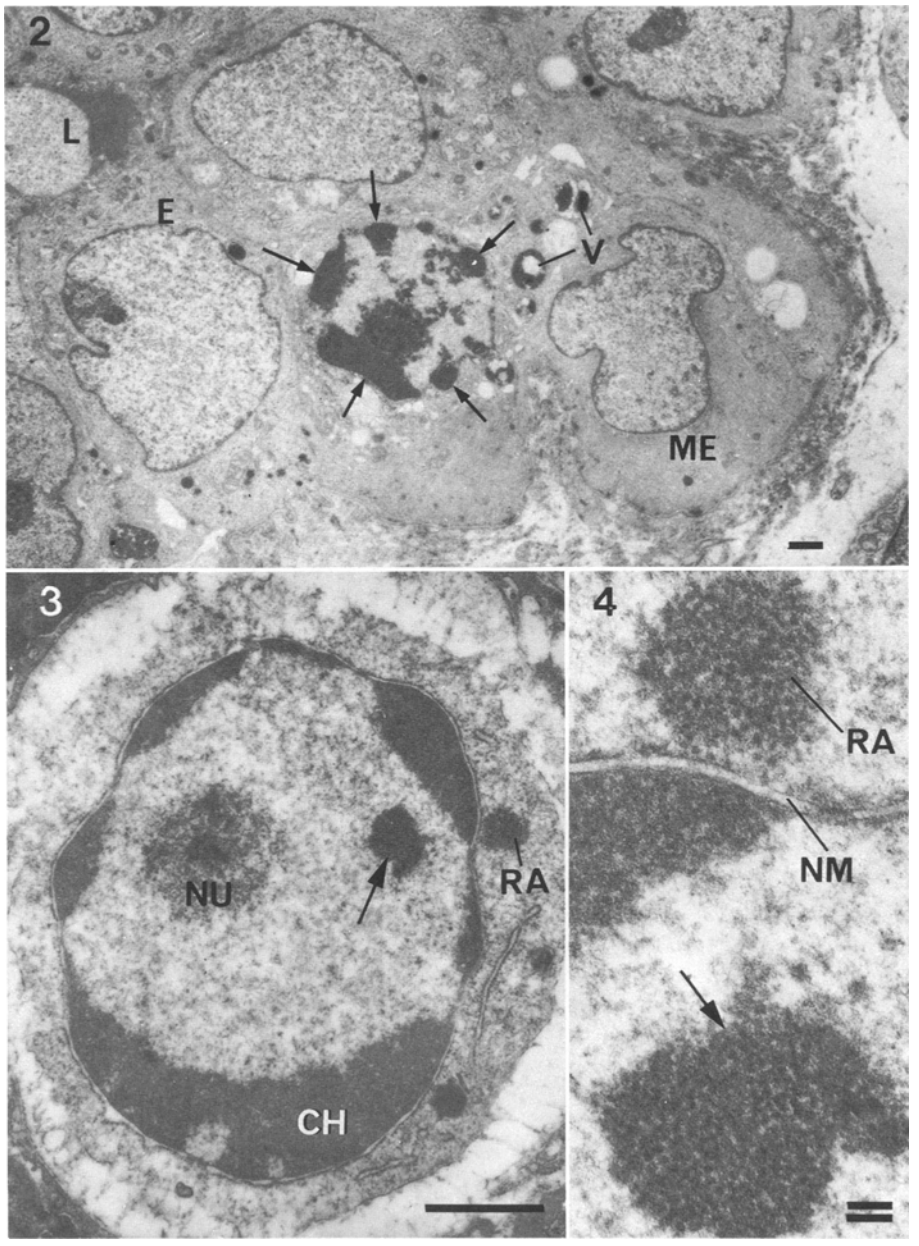

Fig. 2. An early apoptotic cell situated between the myo- and epithelial cells showing peripherally condensed chromatin within the nucleus (*arrows*). ×4,500

Fig. 3. In this apoptotic cell the nucleus contains peripherally condensed chromatin, the remnants of a nucleolus, and a granular aggregate (*arrow*). A small ribosome aggregate is present in the cytoplasm. ×16,000

Fig. 4. An enlargement of part of Figure 3 showing details of the nuclear granular aggregate (*arrow*) and the ribosome aggregate. ×67,500

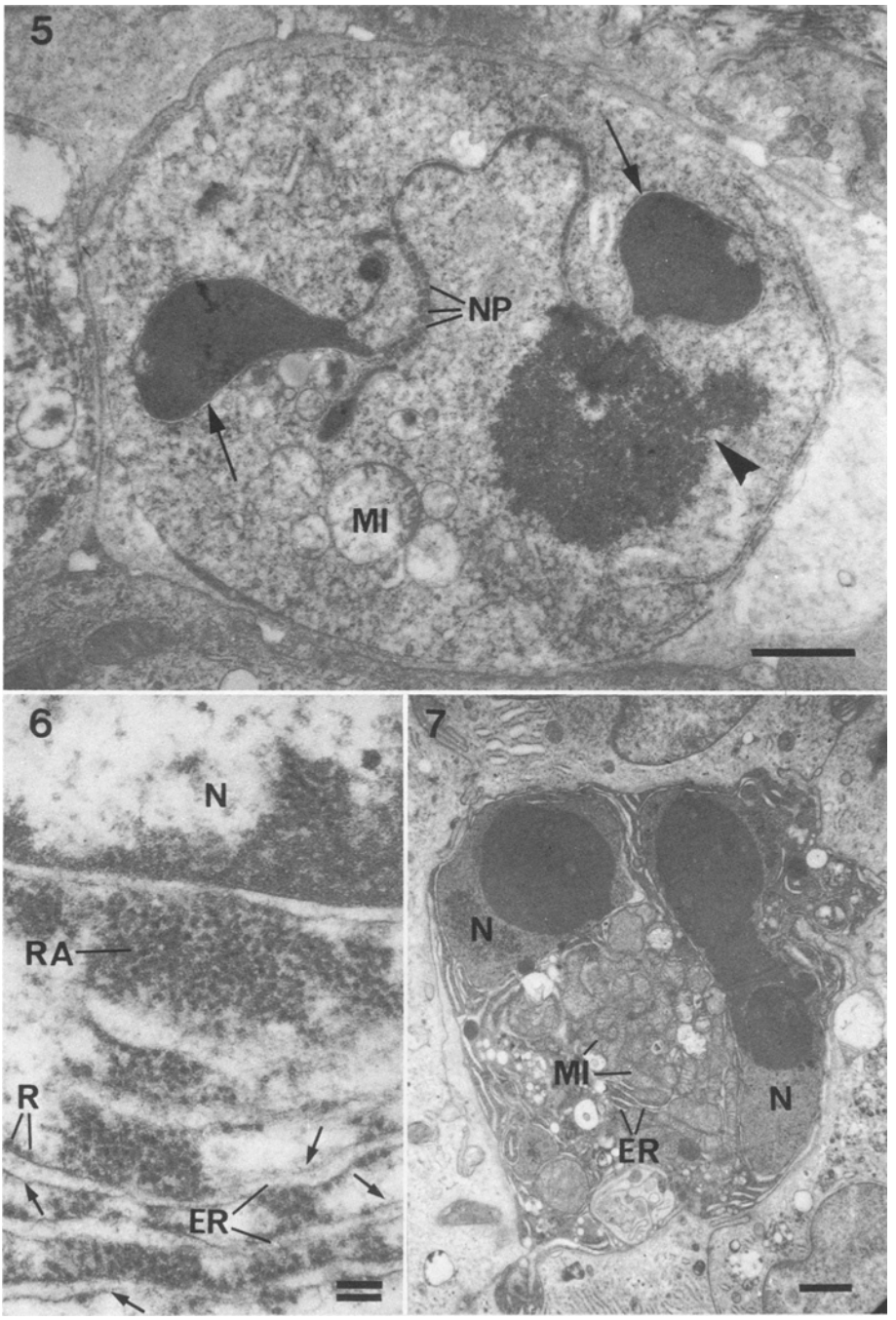

Fig. 5. The nucleus of the apoptotic cell has an irregular outline with the formation of blebs containing dense chromatin (*arrows*). Note the numerous nuclear pores in certain regions of the nuclear membrane and the continuum between the nuclear granular aggregate and the ribosomal aggregate (*arrow head*). ×14,000

Fig. 6. Portion of an early apoptotic cell showing regions of the endoplasmic reticulum free of ribosomes (*arrows*) and aggregations of the free ribosomes. ×62,500

Fig. 7. An apoptotic cell in which two nuclear fragments can be seen. The condensed cytoplasm contains endoplasmic reticulum and numerous normal mitochondria. ×7,300

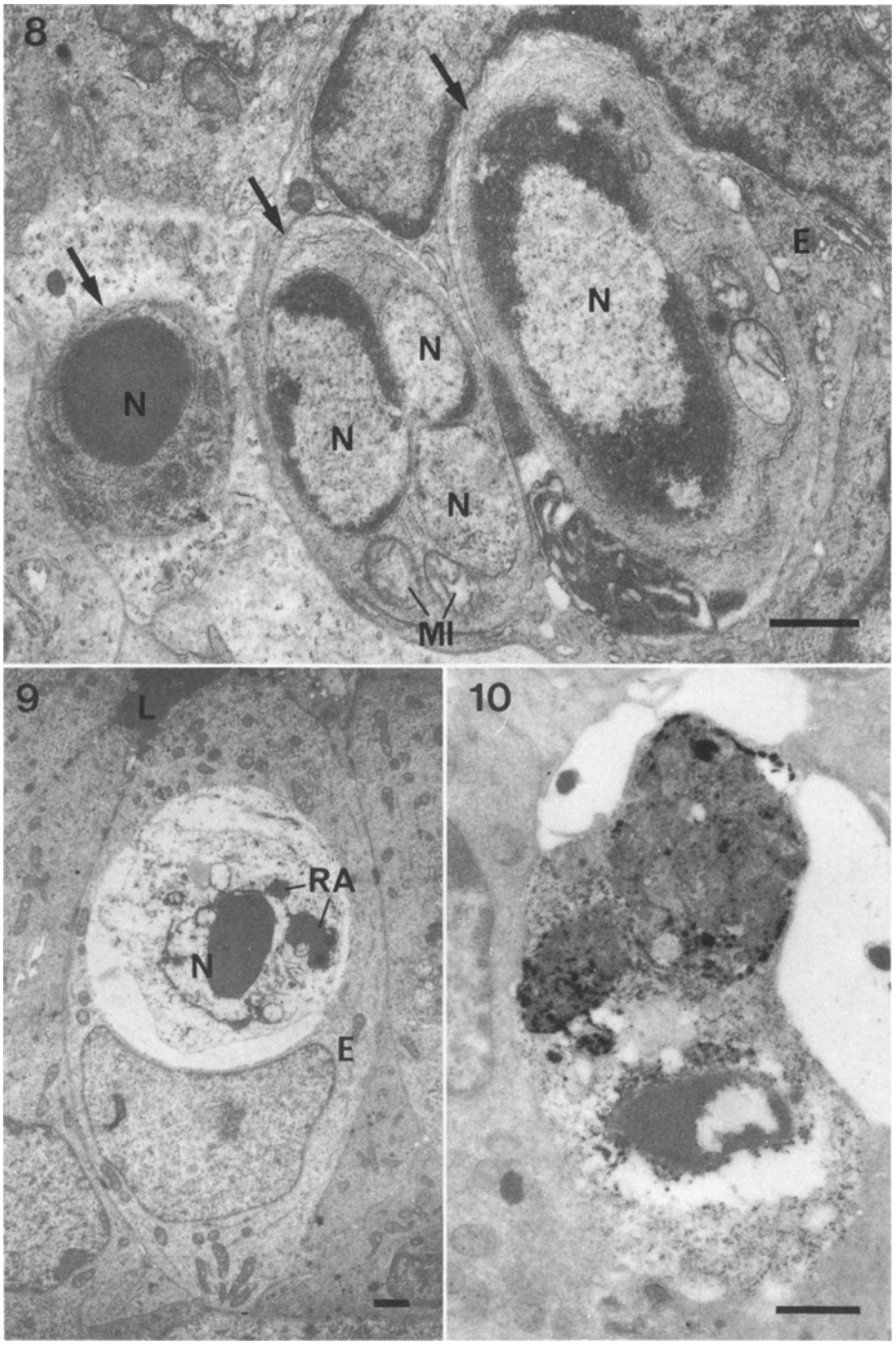

Fig. 8. In this section three apoptotic bodies (*arrows*) can be seen within separate phagosomes, two of which are within the same epithelial cell. × 12,000

Fig. 9. An apoptotic cell within a phagosome of an epithelial cell prior to lysosome digestion. × 4,500

Fig. 10. This section shows that the dense deposits due to acid phosphatase activity are localised within a phagosome containing an apoptotic cell. × 11,000

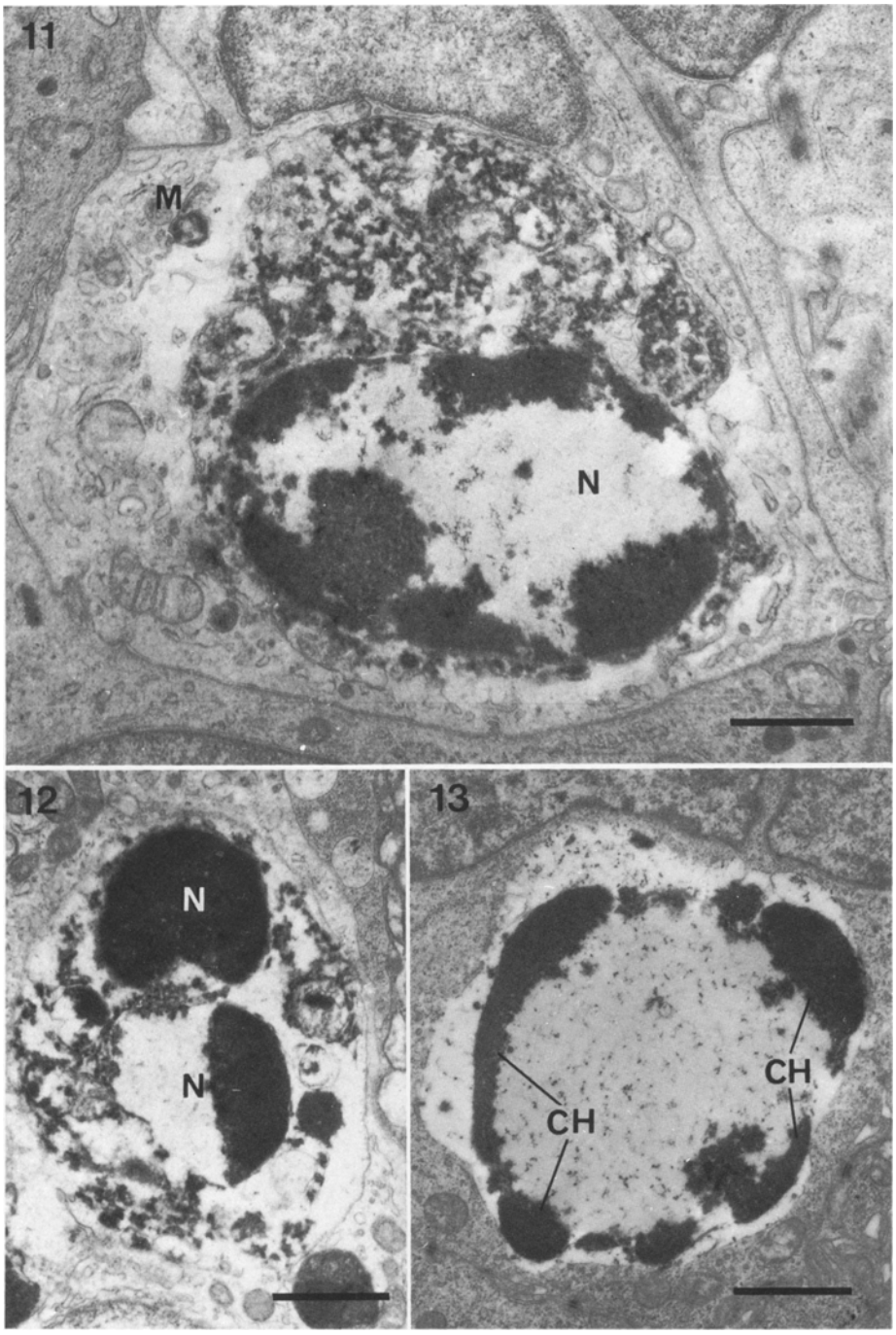

Fig. 11. An apoptotic cell undergoing lysosomal digestion within the phagosome of a macrophage. $\times 17{,}000$

Fig. 12. A phagosome containing an apoptotic cell at an advanced stage of lysosomal digestion in which the nuclear remnants can still be identified. $\times 16{,}000$

Fig. 13. A nuclear ghost of condensed chromatin is present within the phagosome of an epithelial cell. $\times 16{,}000$

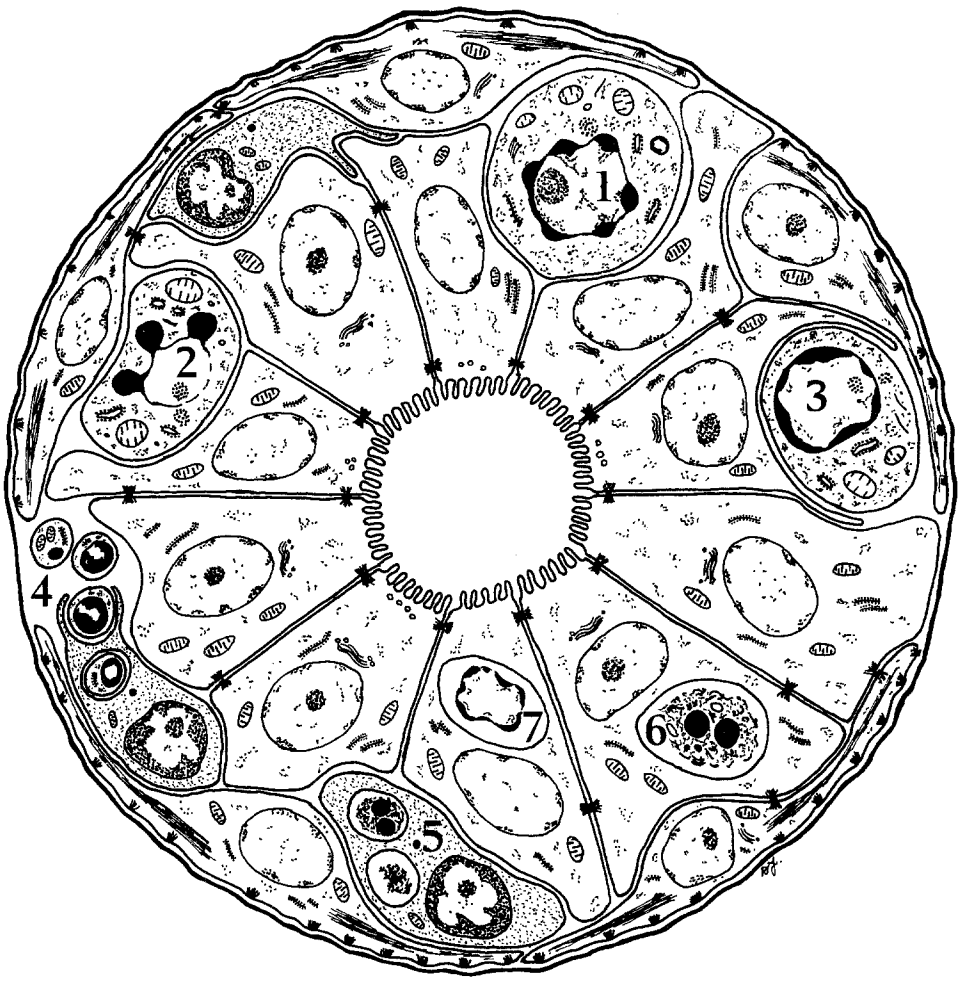

Fig. 14. A diagrammatical summary of the variations in the process of apoptosis observed within the breast epithelium. At the earliest stage of apoptosis recognised the nucleus of the dying cell had peripherally condensed chromatin (*1*). After the initial nuclear changes the cell was either phagocytosed (*3*) and digested within a phagosome (*5, 6, 7*) or underwent nuclear blebbing (*2*) and fragmentation followed by cytoplasmic fragmentation (*4*) prior to phagocytosis and degradation (*5, 6, 7*)

was observed (Fig. 5). The nuclear blebs containing dense chromatin were limited by a nuclear membrane possessing few nuclear pores whereas regions of nuclear membrane free of chromatin aggregates were rich in nuclear pores (Fig. 5). At this stage there were breaks in the nuclear membrane and a continuum was observed between the nuclear granular aggregate and cytoplasmic ribosome aggregate (Fig. 5). This blebbing leads to fragmentation of the nucleus (Fig. 7) which was followed by fragmentation of the cell giving rise to a few (3–4) apoptotic bodies. These bodies were limited by an intact plasmalemma and normally contained one or more nuclear fragments in addition to the cytoplasmic

organelles, including the ribosome aggregates. It would appear that the apoptotic bodies were quickly phagocytosed because the majority were observed within phagosomes (Fig. 8). In addition, it was apparent that phagocytosis could occur at any stage during this process of nuclear and cytoplasmic fragmentation. The majority of cells were phagocytosed by the time of nuclear fragmentation and very few dying cells progressed to the stage of cytoplasmic fragmentation.

Irrespective of the stage at which they were phagocytosed, the apoptotic cells initially retained their integrity (Figs. 8 and 9) prior to undergoing digestion and degradation within the phagosomes (Figs. 11, 12 and 13). The presence of lysosomal enzymes within the phagosomes containing apoptotic cells was confirmed by the cytochemical identification of acid phosphatase activity (Fig. 10). The action of the lysosomal enzymes presumably causes the breakdown of the cytoplasmic organelles and membrane degradation (Figs. 11 and 12). The digested material is absorbed leaving behind a little indigestible debris (Fig. 13). It would appear that the condensed chromatin fragments were most resistant to degradation and could often be identified when other organelles were unrecognisable (Figs. 11 and 12). In certain cases, all that remained within the phagosome was a nuclear ghost consisting of the peripheral chromatin aggregates (Fig. 13). A diagram summarising the variations in the process of apoptosis observed within the breast epithelium is given in Fig. 14.

The morphological changes associated with the intercellular phase of apoptosis (chromatin condensation, nuclear and cytoplasmic fragmentation) would appear to be rapid because relatively few cells were observed at these stages in comparison to the number of cells seen undergoing digestion within phagosomes.

Discussion

In this study we show that the morphological process of cell death occurring within the parenchyma of the "resting" human breast is that of apoptosis. This is characterised by a progression of morphological changes which involve initial nuclear alterations followed by nuclear and cytoplasmic fragmentation. The initial nuclear change involving condensation and peripheral aggregation of the chromatin is found universally in cells undergoing apoptosis (Wyllie et al. 1980). It has been shown in the case of rat cortical thymocytes exhibiting apoptosis, that the nuclear change is associated with activation of an endonuclease (Wyllie 1980). The disintegration of the nucleolus as seen in the present study has been reported in nerve cell deletion in the chick embryo (O'Connor and Wyttenbach 1974; Pannese 1976). However, the aggregation of the granular component of the nucleolus observed in the breast has not previously been reported although such a structure can be seen in Fig. 3a of Pannese (1976). The similarity of the granular component of the nucleolus to ribosomes has previously been noted (review Ghosh 1976). The detachment of the ribosomes from the endoplasmic reticulum and the formation of ribosomal aggregates is similar to the changes observed within dying cells of the chick embryo although the formation of ribosome crystals is not observed in the present study (Bellairs 1961; Mottel and Hammar 1972; O'Connor and Wyttenbach 1974; Pannese

1976). These nucleolar and ribosomal changes are thought to represent a reduction in the dying cell's ability to synthesise RNA and protein.

In the case of the epithelial cells of the breast only a few of the dying cells progress to the stage of nuclear and cytoplasmic fragmentation. This is apparently due to the early recognition and efficient phagocytosis of dying cells. The mechanism by which apoptotic cells are recognised is not fully understood. From in vitro studies with rodent tissue using non-activated peritoneal macrophages and apoptotic thymocytes it would appear that antibody and complement are not involved in recognition of apoptotic cells although a serum factor assists in their phagocytosis (Hargreaves and Wyllie 1981). In the case of the breast epithelium it would appear that the plasmalemmal changes which allow apoptotic cells to be recognised occur at the same time as the early nuclear changes. The cell surface changes are such that neighbouring cells not normally phagocytic (epithelial and myoepithelial cells), and phagocytes (macrophages) can recognise and phagocytose the apoptotic cell.

In the breast, irrespective of the stage of apoptosis at which a cell is phagocytosed the result is lysosomal digestion within the phagosome with ultrastructural changes similar to those described in other tissues (Wyllie et al. 1980). One unusual feature is the resistance of the condensed chromatin aggregates to digestion within the phagosome. Whether this is due to the absence of a specific nuclease or proteinase necessary for the digestion of the aggregates is unknown.

The stimuli which cause cells to undergo apoptosis are extremely varied but it is known that physiological changes can initiate apoptosis under certain circumstances. This led to the hypothesis that it is the morphological process involved in "programmed cell death". It has been shown that the incidence of apoptosis in hormone dependent tissue could be related to changes in hormone levels (Wyllie et al. 1980). In the case of the breast, the parenchyma is known to be responsive to the hormones oestrogen and progesterone, a characteristic shared with the endometrium. In the hamster endometrium it would appear that apoptosis is initiated by a reduction in the nuclear oestrogen receptor concentration which can be brought about by decreases in the oestrogen level or by increases in the progesterone level (West et al. 1978; Sandow et al. 1979). We have examined the incidence of apoptosis in the "resting" breast in relation to the menstrual cycle and found increased apoptosis just prior to and during menses (Ferguson and Anderson 1981), which is similar to that reported for the endometrium (Hopwood and Levison 1976). Therefore in the case of the human breast, apoptosis would appear to be a response to the decreasing hormone levels which occur towards the end of the menstrual cycle (Ferguson and Anderson 1981).

In the "resting" breast, the elevated levels of apoptosis are preceded by elevated levels of mitosis and it would appear that apoptosis is the mechanism by which cell proliferation is balanced by cell deletion (Ferguson and Anderson 1981). Therefore apoptosis has an important role to play in preserving the normal parenchymal architecture of the breast.

Acknowledgements. The co-operation of the Clinical Staff of the Surgical Unit, Longmore Hospital, and the technical assistance of Mrs M Hearn, Mrs M McHenry and Mr S McKenzie is gratefully acknowledged. This work was funded by Project Grant G978/336 to TJA from the Medical Research Council.

References

Barka T, Anderson PJ (1962) Histochemical methods for acid phosphatase using hexazonium pararosinilin as coupler. J Histochem Cytochem 10:741–753

Bellairs R (1961) Cell death in chick embryos as studied by electron microscopy. J Anat 95:54–60

Ferguson DJP, Anderson TJ (1981) Morphological evaluation of cell turnover in relation to the menstrual cycle in the "resting" human breast. Br J Cancer (in press)

Ghosh S (1976) The nucleolar structure. Int Rev Cytol 44:1–28

Hargreaves AD, Wyllie AH (1981) Recognition of apoptotic thymocytes by macrophages. Meeting Pathological Society of Great Britain and Ireland. London Jan 1981. Abstract: J Pathol (in press)

Hopwood D, Levison DA (1976) Atrophy and apoptosis in the cyclical human endometrium. J Pathol 119:159–166

Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 26:239–257

Mottet NK, Hammer SP (1972) Ribosome crystals in necrotizing cells from the posterior necrotic zone of the developing chick limb. J Cell Sci 11:403–414

O'Connor TM, Wyttenbach CR (1974) Cell death in the embryonic chick spinal cord. J Cell Biol 60:448–459

Ozzello L (1971) Ultrastructure of the human mammary gland. In: Sommers SC (ed) Pathology annual. Appleton-Century-Crofts, New York, pp 1–59

Pannese E (1976) An electron microscopic study of cell degeneration in chick embryo spinal ganglia. Neuropathol Appl Neurobiol 2:247–267

Sandow BA, West NB, Norman RL, Brenner RM (1979) Hormonal control of apoptosis in hamster uterine luminal epithelium. Am J Anat 156:15–36

Stirling JW, Chandler JA (1976) The fine structure of the normal resting terminal ductal lobular unit of the female breast. Virchows Arch [Pathol Anat] 372:205–226

Stirling JW, Chandler JA (1977) The fine structure of ducts and subareolar ducts in the resting gland of the female breast. Virchows Arch [Pathol Anat] 373:119–132

Tannenbaum M, Weiss M, Marx AJ (1969) Ultrastructure of the human mammary ductule. Cancer 23:958–978

Trump BF, Mergner WJ (1974) Cell injury. In: Zweifach BW, Grant L, McCluskey RT (eds) The inflammatory process, 2nd edn, vol 2. Academic Press, New York, pp 115–257

West NB, Norman RL, Sandow BA, Brenner RM (1978) Hormonal control of nuclear estrodiol receptor content and the luminal epithelium in the uterus of the golden hamster. Endocrinology 103:1732–1741

Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555–556

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306

Accepted July 14, 1981