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Bio-morphological events in the development of the human female mammary gland from fetal age to puberty

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Abstract Bio-morphological understanding of the developing human mammary glands may clarify some aspects of breast pathology, including cancer. In particular, some epidemiological data suggests that during fetal growth an altered intrauterine hormonal status, especially a change in estrogen status, could predispose to carcinogenesis. In an attempt to achieve new information on early breast growth, a series of developing human breasts have been analyzed, namely: 4 fetal breasts (28–32 weeks of gestational age), 7 infant breasts (7 h to 2 years) and 1 puberal breast (12 years). In addition to the morphological features, we studied the immunohistochemical expression of some markers involved in morphogenesis, such as MIB-1 for cell proliferation, bcl-2 for apoptosis control, CD34 for vasculogenesis, estrogen (ER) and progesterone (PR) receptors for hormonal profile, and smooth-muscle actin for myoepithelial differentiation. The results were as follows: (a) lobules, absent between 28 weeks and 2 days, were well evident at 2 years of age and at puberty; (b) myoepithelial cells appeared from 28 weeks onward and persisted later with no modification in quantity and distribution; (c) epithelial cell proliferation was constantly low; (d) in all breasts inner epithelial cells showed diffuse bcl-2 positivity, while basal myoepithelial-like cells were generally negative; (e) all breasts were well vascularized with two different patterns: periductal vascularization (PDV) and interductal vascularization (IDV), IDV being always present, whereas PDV was found only in infant breasts; (f) ER and PR were almost absent in fetal and infant breasts, while their expression was high in the epithelial cells of the puberal breast; (g) stromal cells had no hormonal receptors and were heterogeneous for proliferation and bcl-2 expression. Interestingly, two fetal breasts showed high proliferation and high ER expression, respectively, in their epithelial compartment. This could be the expression of an altered hormonal environment in utero, representing a basis for possible subsequent cancer initiation.

Key words Breast development · Human breast · Fetal breast · Immunohistochemistry

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Introduction

The knowledge of the events underlying the differentiation and morphogenesis of the human female breast may clarify some features concerning the anatomy of the normal adult breast and the pathogenesis of breast lesions [25]. Immunohistochemical and ultrastructural studies on the developing breast have been able to explain some aspects of neuroendocrine [29] and apocrine [30] differentiation in the adult mammary gland.

The morphological steps in human mammary gland development are complex and still not completely clear; however, some major phases of glandular tree growth have been described in utero, during the first 2 years of life and at puberty [3, 19].

The structural development of the ductal system and the functional differentiation of the lining epithelium in the first phase of development have been described and division of breast growth into three morphological types (MT I–III) and five functional stages (FS I–V) proposed [3]. Furthermore, an immunohistochemical analysis concerning the distribution of cytoskeletal proteins and other molecules has clarified some aspects of epithelial differentiation and of the relationship between epithelium and stroma during breast development [4, 5, 22].

Study of the events playing a relevant role in morphogenesis could offer further information about the understanding of early breast growth, namely cell proliferation, apoptosis control, vascularization and hormonal responsiveness.

In this study the expression of some proteins involved in the above events, MIB-1 for cell proliferation, bcl-2 for apoptosis control, CD34 for vascularization and estrogen and progesterone receptors for the hormonal profile in fetal, infant and puberal human female breasts was investigated. In addition, myoepithelial differentiation was also analyzed by a myoepithelial marker as smooth-muscle actin.

Materials and methods

Specimens

Twelve autoptic female breasts were obtained after parental consent. They were:

1. 4 Fetal breasts from fetuses of 28, 29, 29 and 32 weeks' gestational age;
2. 7 Term infant breasts from infants that had died at 7, 8, 12 and 18 h, 1 and 2 days, and 2 years of age;
3. 1 Puberal breast from a 12-year-old.

The causes of death are reported in Table 1. The nipple-areola and surrounding tissue were excised, fixed in formalin, and embedded in paraffin. Sections (4 μm) of each breast were stained with hematoxylin-eosin for morphological evaluation. Additional 4-μm sections were used for immunohistochemical study.

Proteins and antibodies

Cell proliferation was checked with the aid of MIB-1, an antibody directed against recombinant parts of ki-67 [8], a nuclear antigen expressed in all phases of the cell cycle except for G_0 and early G_1 [14], used as a cell proliferation marker (DBA, dilution 1:200).

For apoptosis control an anti-bcl-2 antibody was used (MAb 124, DBA, dilution 1:100). bcl-2, functioning as a cell death suppressor, could promote tissue growth by reducing the cellular loss rate [16]; there is evidence that it is involved in the differentiation and morphogenesis of several tissues, including fetal breast [21, 23].

Vascularization was studied by means of an anti-CD34 antibody (QB-END10, Novocastra Lab., dilution 1:100); vascularization is a prominent event during embryonic development and somatic growth [13], playing an important part in the complex mechanisms that regulate the interactions between mesenchymal and epithelial structures. The immunohistochemical expression of CD34, a protein associated with vascular endothelial cells, permits the evaluation of microvessel distribution and density [24].

Table 1 Age and cause of death of each fetus and infant (*GA* gestational age)

	Age	Cause of death
Fetus	28 weeks GA	Chorioamnionitis
	29 weeks GA	Chorioamnionitis
	29 weeks GA	Placental hemorrhage
	32 weeks GA	Unknown
Infant	7 h	Pneumonia
	8 h	Pneumonia
	12 h	Pneumonia
	18 h	Cerebral hemorrhage
	1 day	Pneumonia
	2 days	Hyaline membrane disease
	2 years	Pneumonia
Puberal girl	12 years	Cerebral hemorrhage

Hormonal receptors: hormonal responsiveness, a critical aspect of the mammary gland, has been documented in the adult breast [7, 18, 26], but data concerning the role of hormones in fetal breast development has been reported only rarely [9, 10]. Antibodies against estrogen (ER88, Menarini-BioGenex, dilution 1:30) and progesterone (PR88, Menarini-BioGenex dilution 1:30) receptors have been used to analyze the distribution of these receptors in the ductal-stromal compartment of the developing human breast.

Myoepithelial differentiation was studied by means of an antibody against α-smooth muscle actin (1A4, DBA dilution 1:50), a marker of myoepithelial differentiation in both adult [15] and infant breasts [4].

Method

The sections were deparaffinized in xylene and rehydrated in alcohols. Endogenous peroxide activity was blocked by incubating the slides in 1% hydrogen peroxide in methanol for 10 min. Tissue sections stained for MIB-1 were pretreated with 0.1% trypsin and 0.1% calcium chloride buffered to pH 7.6. To unmask the antigens, the slides, except for actin, were microwaved in 10 mM citrate buffer, pH 6, for a total of 10 min. After nonspecific staining had been blocked with normal horse serum, the sections were incubated with primary antibodies. Incubation time was 12 h at 4°C for bcl-2, MIB-1 and CD34, and 1 h at room temperature for ER, PR and actin. The sections were then incubated with biotin-labelled secondary antibody (dilution 1:500) and avidin–biotin complex (Vector Burlingame, Calif.) for 30 min each. 3–3' Diaminobenzidine tetrahydrochloride with 0.01% hydrogen peroxide was used as chromogen. The alkaline phosphatase–anti-alkaline phosphatase (APAAP) method was used to amplify the bcl-2 signal. The reaction was developed with alkaline phosphatase containing naphthol, AS-MX, fast red and levamisol (APAAP kits DAKO, Milan, Italy).

Controls

Positive controls were included in each staining and consisted in breast invasive carcinomas known to express MIB-1, ER and PR. Positive control for bcl-2 was follicular non-Hodgkin lymphoma. Vascular endothelial cells were used as a positive internal control for CD34. Positive control for α-smooth muscle actin was a section of normal adult breast parenchyma. Negative controls were obtained by omitting primary antibodies.

Evaluation parameters

Nuclear staining was considered positive for MIB-1, ER and PR and cytoplasmic staining for bcl-2 and actin. The distribution of positivity for MIB-1, bcl-2, ER, and PR was evaluated in both the epithelial and stromal compartment. The grade of positivity was evaluated semiquantitatively by counting a minimum of 300 cells and calculating the percentage of immunoreactive cells.

With regard to vascularization the presence of two distinct patterns was demonstrated and will be reported in detail in "Results"; the two patterns have been named periductal vascularization (PDV) and interductal vascularization (IDV). PDV was defined by microvessel cuffing in immediate apposition to the basement membrane of the ducts; PDV was quantified as: – (no ducts with PDV), + (some ducts with PDV) and ++ (most ducts with PDV). IDV consisted in the microvessels of the stroma around and amongst the ducts except for periductal vascular cuffing; IDV was quantified as microvascular density (MDV) by counting the microvessels at ×200 magnification (×20 objective lens and ×10 ocular lens; 0.74 mm^2 per field). In each case the microvessels of three different vascular fields were recorded and averaged. A single microvessel was defined as a discrete cluster of cells stained for CD34; the presence of a lumen was not required for scoring as a microvessel.

Table 2 MIB-1, bcl-2, estrogen receptor (ER), progesterone receptor (PR) and actin expression in epithelial cells and microvessel distribution of the fetal, infantile and puberal female human breasts (*%* percentage of positive cells, *PDV* periductal vascularization [– absent, + moderate, ++ diffuse], *MVD* microvascular density [+ <371, ++ >371], *ME(+)* positive myoepithelial cells)

Age	Markers					
	MIB-1 (%)	bcl-2 (%)	ER (%)	PR (%)	Vessels (CD34)	Actin
Fetal breasts						
28 weeks	<1	60	<1	<1	PDV(–) MVD(+)	ME(+)
29 weeks	10	70	<1	<1	PDV(–) MVD(+)	ME(+)
29 weeks	<1	70	<1	<1	PDV(–) MVD(+)	ME(+)
32 weeks	<1	80	10	<1	PDV(–) MVD(++)	ME(+)
Infant breasts						
7 h	1	40	<1	<1	PDV(+) MVD(+)	ME(+)
8 h	1	60	<1	<1	PDV(++) MVD(++)	ME(+)
12 h	1	40	<1	<1	PDV(+) MVD(++)	ME(+)
18 h	1	50	<1	<1	PDV(+) MVD(++)	ME(+)
1 day	1	50	<1	<1	PDV(+) MVD(++)	ME(+)
2 days	1	50	<1	<1	PDV(+) MVD(+)	ME(+)
2 years	1	50	<1	<1	PDV(++) MVD(++)	ME(+)
Puberal girl						
12 years	1	50	50	50	PDV(–) MVD(+)	ME(+)

Results

Breast structure

1. Fetal breasts (28–32 weeks) appeared as nodular areas of variable size, formed of a mixture of epithelial structures and mesenchyma extending from the epidermis to the subcutaneous tissue

   Glandular tree. All breasts showed round-oval and elongated ducts, sometimes with branching and round end-buds. The lumina of some of the ducts contained amorphous eosinophilic material, desquamated cells and necrotic debris. No lobular structure was found (Fig. 1)

   Epithelium. The ducts were lined with two or three layers of epithelial cells; diffuse areas of cell multilayering with irregular secondary lumina were also observed. The cells close to the basement membrane often showed clear cytoplasm and indistinct borders, while the cells forming the inner layers of the ducts were often weakly or moderately eosinophilic (Fig. 2)

   Stroma. The periductal stroma was loose and richly cellular; subtle strands of dense connective tissue were present in the interductal site

2. Infant breasts (7 h to 2 days)

   Glandular tree. The distribution and morphology of the ducts were similar to those in the fetal breasts; in two breasts (18 h, 1 day) some ducts showed cystic dilatation (Fig. 3)

   Epithelium. The cytological features were similar to those of fetal breasts

   Stroma. The characteristics of the stroma were similar to those in the fetal breasts

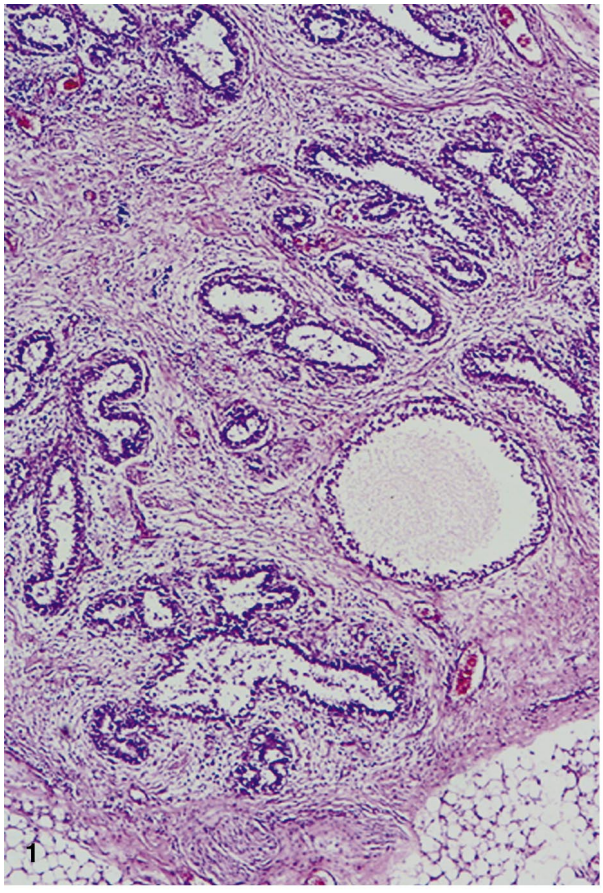
3. Infant breast (2 years) and puberal breast (12 years)

   Glandular tree. The two breasts were formed by round to oval ducts with blunted borders and more rarely by elongated ducts with three or four terminal branching ductules forming rudimental lobules (Fig. 4)

   Epithelium. The ducts were generally lined with two or three layers of cells. The inner cells were cuboidal-cylindrical with medium-size nuclei and weakly eosinophilic cytoplasm; the outer cells were cuboidal with oval nuclei. Cell multilayering was rarely observed

   Stroma. The connective tissue was diffusely dense

The levels of MIB-1, bcl-2, ER, PR and actin expression observed in the epithelial and stromal cells of fetal, infant and puberal breasts are summarized in Tables 2 and 3. Vascular distribution data is also given in Table 2.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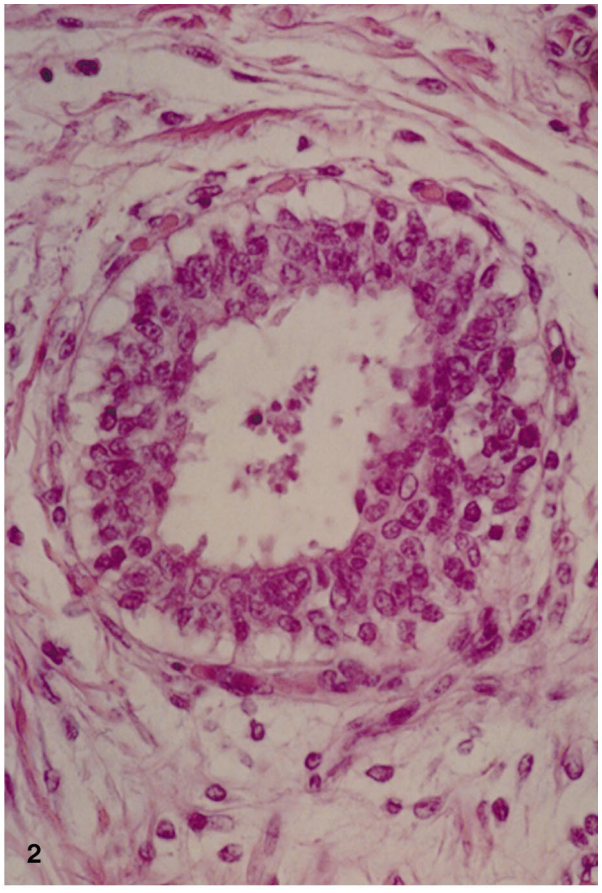
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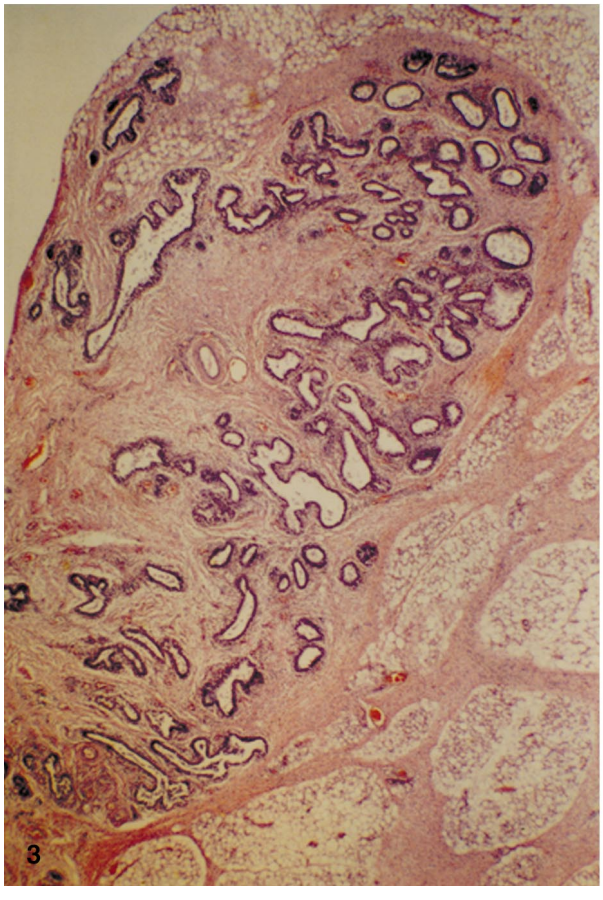
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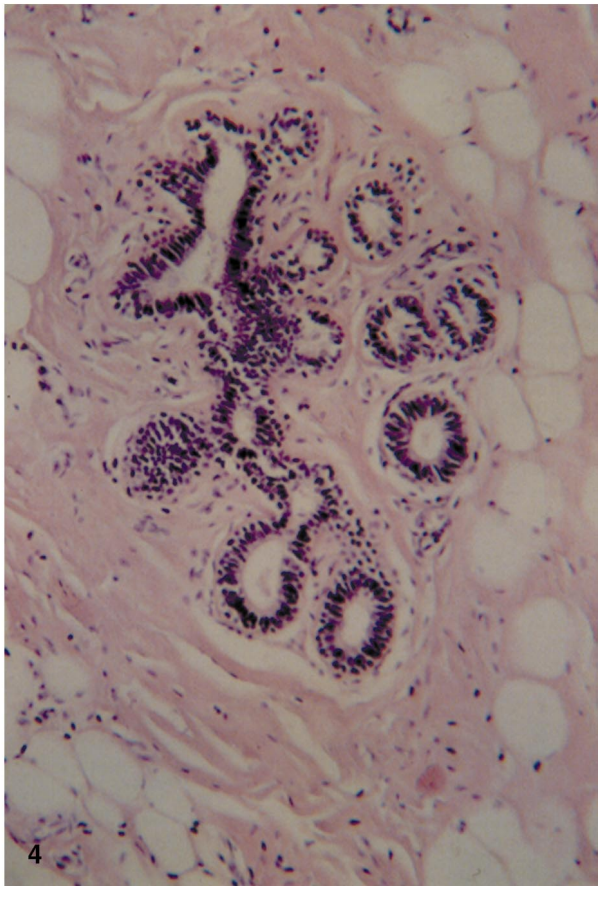
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Table 3 MIB-1, bcl-2, ER and PR immunoreactivity in stromal cells of fetal, infant and puberal human breast tissues (++ numerous positive cells, + low number of positive cells, +/– rare positive cells, – absence of positive cells)

Age	Stromal cells			
	MIB-1	bcl-2	ER	PR
28 weeks	++	+	–	–
29 weeks	++	+	–	–
29 weeks	++	+/–	–	–
32 weeks	++	+	–	–
7 h	+	+	–	–
8 h	++	+/–	–	–
12 h	+	+/–	–	–
18 h	+	+	–	–
1 day	+	+/–	–	–
2 days	+	+/–	–	–
2 years	+	+/–	–	–
12 years	+/–	+/–	–	–

Cell proliferation was investigated. In fetal and infant breasts the number of MIB-1-positive epithelial cells was low (≤1%) and the immunoreactive cells were mainly identified in the inner epithelial layer (Fig. 5). In a 29-week breast a higher number of MIB-1-positive cells (10%) was observed in the innermost layer of several ducts. The puberal breast showed low cell proliferation (1%). A variable number of MIB-1-positive stromal cells was found in all breasts. MIB-1 positivity was more diffuse in fetal breasts.

When apoptosis control was monitored, all breasts were found to have a relatively high proportion of bcl-2-positive epithelial cells, ranging between 40% and 80%. The bcl-2 positivity was localized in the inner epithelial layers, while basal cells were substantially negative (Fig. 6). Rare bcl-2 positive stromal cells were present in all breasts.

Two distinct vascularization patterns, defined as PDV and IDV, were observed. Complete PDV, in which microvessels form a continuous rim around the circumference of the majority of ducts (Fig. 7), was observed in two breasts (8 h and 2 years). In five breasts (7 h, 18 h, 12 h, 1 day and 2 days) PDV was less evident, with only a few ducts partially or totally surrounded by a rim of microvessels. The other breasts showed no PDV. IDV was characterized by a delicate diffuse stromal vascular framework, mostly localized around the ducts. The quantification of this vascularization, as defined by MVD, evidenced high values with a range of microvessels between 320 and 470 (mean 368; median 371).

Hormonal receptors were sought. The puberal breast showed numerous PR- and ER-positive epithelial cells (50%) in the inner layers of all ducts (Fig. 8). On the other hand, fetal and infant breasts were substantially lacking both types of hormonal receptors; in fact a very low number of PR- and ER-positive cells (<1%) was found in the inner layers of some ducts. Interestingly, a fetal breast of 32 weeks showed a focal ER immunoprofile similar to the puberal breast, characterized by the presence of numerous positive cells in the inner layer of some ducts (Fig. 9).

Myoepithelial differentiation was checked, and in all breasts smooth-muscle actin immunoreactivity was detected in most of the basal myoepithelial-like cells of the ducts.

General conclusions

1. All the breasts taken between 28 weeks of gestation and 2 days of life were devoid of lobules, having only ductal structures surrounded by loose stroma and with frequent cell multilayering; a number of lobules, although still rudimentary, were recognized in the 2-year-old's breast and in the puberal breast, associated with the reduction of cell multilayering in the ducts and dense periductal connective tissue.
2. Basal myoepithelial cells were well represented at all ages, with no modification in quantity and distribution.
3. The proliferation rate of the epithelial cells was consistently low.
4. Diffuse bcl-2 positivity was found in inner epithelial layers of ducts of all breasts; basal myoepithelial-like cells were generally negative.
5. All breasts showed high MVD; infant breasts showed complete or partial PDV.
6. ER and PR expression was high in the puberal breast and very low in infant and fetal breasts.
7. Stromal cells had no hormonal receptors and were heterogeneous for proliferation and bcl-2 expression.

◀ **Fig. 1** Breast from a 29-week fetus. The gland is formed of roundish and elongated ducts. Lobules are absent. Hematoxylin-eosin, ×100

Fig. 2 Breast from a 32-week fetus. The ducts are lined by outer cells with clear cytoplasm and inner, moderately eosinophilic, cells. Periductal stroma is loose. Hematoxylin-eosin, ×250

Fig. 3 Human breast from a full-term neonate who had died 1 day after birth. The gland is formed of round, elongated and cystic ducts. No lobules are in evidence. Hematoxylin-eosin, ×40

Fig. 4 Breast from a 2-year-old who had been born at term. A rudimentary lobular structure with dense stroma. Hematoxylin-eosin, ×250

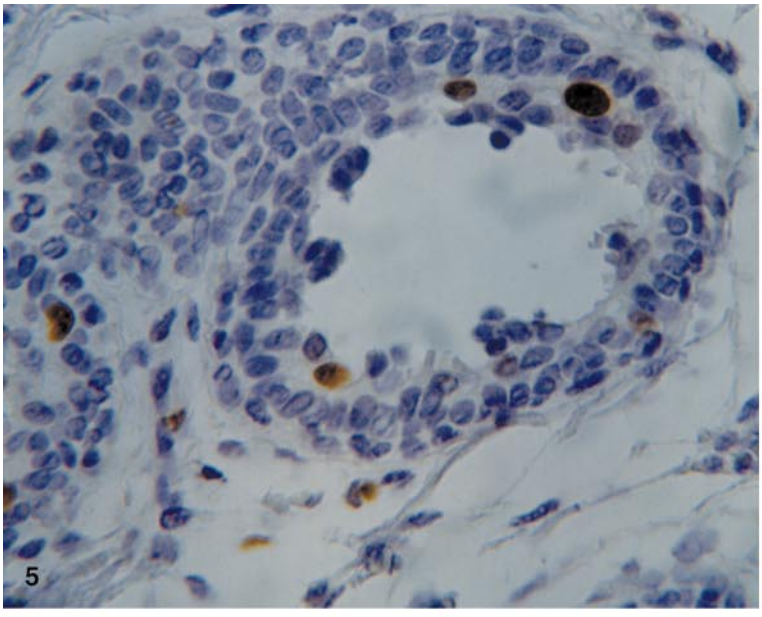
Fig. 5 Breast from a 29-week fetus. A duct with low proliferative activity. MIB-1, ×250 ▶

Fig. 6 Breast from a 29-week fetus. A duct with inner epithelial cells immunoreactive for bcl-2. ×200

Fig. 7 Breast of an infant who had died 8 h after birth at full term. The ducts are totally surrounded by a rim of microvessels. CD34, ×200

Fig. 8 Breast from a 12-year-old. Numerous epithelial cells show nuclear positivity for progesterone receptors. ×250

Fig. 9 Breast from a fetus following death at 32 weeks of gestational age. A duct with diffuse positivity for estrogen receptors. ×250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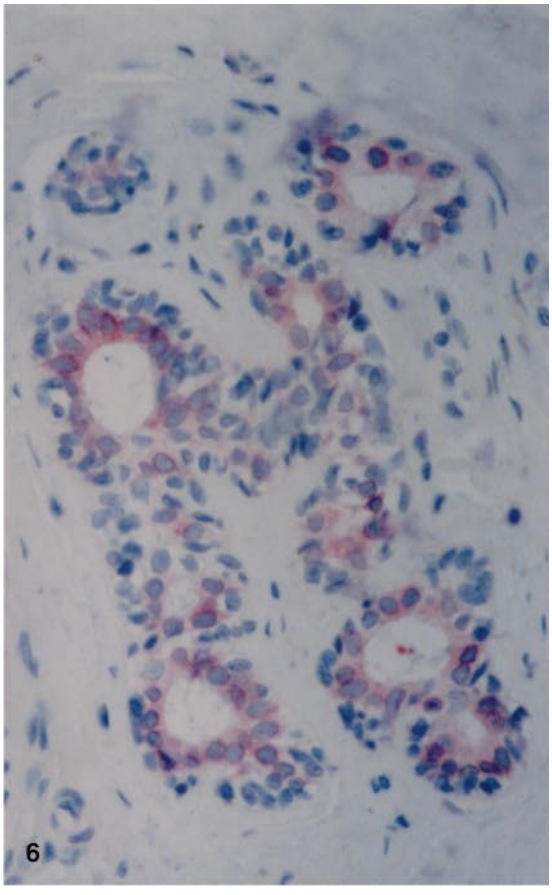
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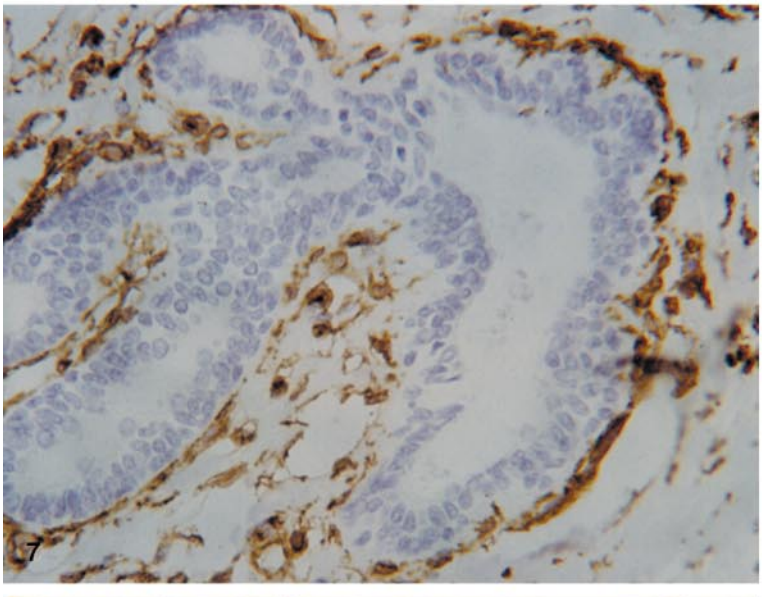
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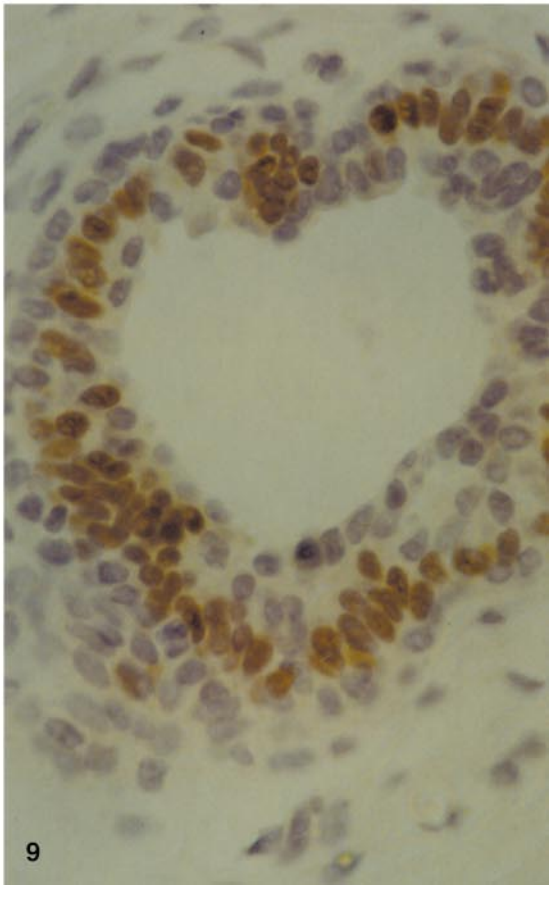
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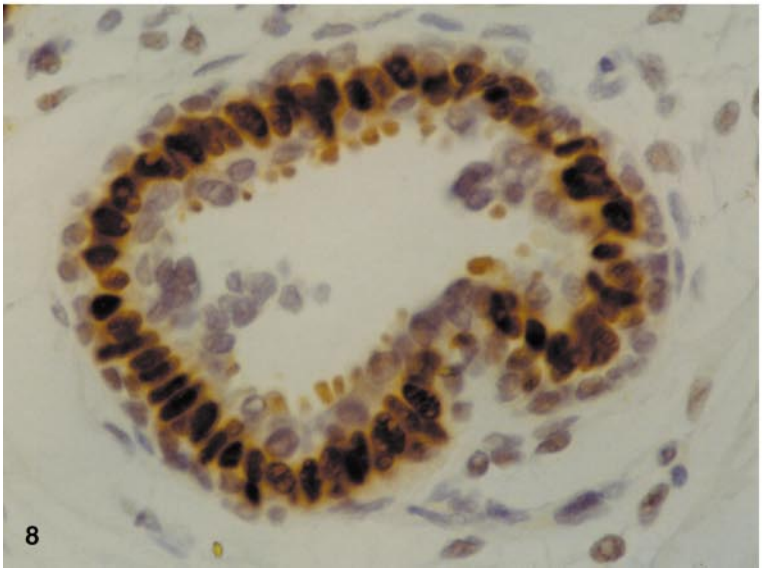
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8. A breast from a 29-week fetus showed focal high epithelial cell proliferation and the breast from the 32-week fetus showed focal puberal-like ER expression.

Discussion

Breast development is a multistep process characterized by complex mesenchymal–epithelia interactions. This process has been partially clarified in some animal models [11], but few data is available on the human breast. It appears important to know about the events involved in human breast development, as there is some evidence that breast cancer initiation could occur during the early phases of growth [1].

The fetal period and the first 2 years of life appear to be crucial for some steps in human breast morphogenesis and cytodifferentiation in that epithelial and mesenchymal components undergo morphological [2, 3] and functional modifications involving modulation of cytokeratins [4], myoepithelial markers [4], bcl-2 expression [21], growth factors and extracellular matrix proteins [22].

In our series we observed some differences between the group of 28-week to 2-day breasts and the 2- and 12-year-old breasts; the latter showed remodelling of the gland compared with the former group, with the presence of rudimentary lobules surrounded by dense connective tissue and reduction of epithelial cell multilayering. Anbhazagan et al. [3], in a series of human breasts from infants between 1 day and 2 years of age, found evidence to show that the ductal system had a variable morphology not correlated with the age of the infant, while epithelial cells underwent chronologically correlated modifications.

From the analysis of our data and that of Anbhazagan et al. [3], it appears that the first 2 years of life are a critical period for some aspects of breast maturation. Again, the gland seems to remain rather stable from 2 to 12 years of age, as indicated by the similar morphology in the breasts from children of these ages.

Actin, a marker of myoepithelial differentiation, was found to be expressed in the basal cells of all breasts, including that taken from the fetus of 28 weeks' gestational age. As, in another study [4], no actin expression was found in a 23-week breast, it appears evident that myoepithelial differentiation occurs in the period ranging from 24 to 28 weeks.

Interestingly, the period of myoepithelial differentiation seems to coincide with that of the modulation of the bcl-2 expression. The inner epithelial layers of all breasts were found to express bcl-2, while basal myoepithelial cells were generally negative. Nathan et al. [21] found bcl-2 expression in the basal epithelial cell layers of breasts of babies between 16 and 23 weeks of age and in the luminal cells of infant breasts. Comparison of our data with the results of Nathan et al. [21] seems to confirm that the period between 24 and 28 weeks is crucial for both bcl-2 modulation and myoepithelial differentiation. The role of bcl-2 has not yet been fully elucidated, but it probably prolongs the life span of multipotential cells and favors the progression of differentiation by preventing cell death. The bcl-2-negative myoepithelial-like cells could represent cells with terminal differentiation.

The epithelial compartment of fetal and infant breasts appears to be essentially a system characterized by very low ER/PR expression and proliferation, suggesting that estrogen and progesterone do not play a predominant role in early development. On the other hand, this fact is in keeping with the in vitro studies of the mouse breast model, in which hormones such as insulin, prolactin and aldosterone, unlike estrogen and progesterone, seem to be heavily involved in early growth [9, 10]. On the other hand, low cell proliferation was also demonstrated in the mouse model, in which the mitotic index of the fetal breast gland turned out to be lower than that of the surrounding epidermis [6]. The puberal breast showed high ER and PR expression, which is probably the consequence of the intense hormonal stimulation to which the breast is exposed in this phase of growth. A high level of hormonal receptors in the puberal breast associated with low proliferation seems to be rather unusual. However, this is not a surprising event, since the relationship between estrogen/progesterone receptor expression and proliferative activity is controversial [17, 27]. In particular, it is not clear whether the immunohistochemical pattern of hormonal receptors corresponds to their real activity, which could suggest that only certain patterns of expression indicate biological activity [7].

All breasts were intensely vascularized, especially in the areas around the ducts. In infant breasts a subtle rim of vessels surrounded several developing ducts either partially or totally, but the same features were not observed in fetal and puberal breasts. The formation of vessels in close contact with the basement membrane could represent a critical step for the maturation of rudimentary ducts, also taking into consideration that the formation of lobules may start to occur in this period. As vasculogenesis is regulated by several factors, the study of these markers could clarify further features of breast vascularization.

The stromal compartment of developing breasts is also subject to remodelling. A reduction in cellularity and an increase in dense periductal connective tissue is observed in the breast from the 2-year-old and in the puberal breast compared with the fetal breasts. There is a correlation between this morphological aspect and the proliferative activity of stromal cells, which is higher in fetal breasts than in infant and puberal breasts. No positivity was found for hormonal receptors in stromal cells, in contrast to the mesenchyme of mouse fetal breast [20].

Two fetal breasts showed focal areas with high ER expression and proliferation, respectively. The complexity of hormonal interactions between fetal and maternal compartments makes it difficult to establish whether these features were caused by particular maternal hormonal environment or whether they reflected an intrinsic potentiality of the epithelial cells. Interestingly, some ep-

idemiological data supports the hypothesis that an altered maternal hormonal status, in particular that of estrogen, during fetal growth could predispose to breast cancer in adult life [1, 28]. The mechanisms involved in this phenomenon are unknown, but high concentrations of maternal hormones in pregnancy probably create the so-called fertile soil for subsequent cancer initiation in the breasts of offsprings [28]. We cannot exclude the possibility that high ER expression and proliferation in two fetal breasts is the expression of an altered hormonal stimulation in utero, but whether or not this is in any way related to cancer initiation remains to be clarified.

Another noteworthy feature concerns the PDV pattern we detected in infant breasts, as a similar vascular pattern has also been observed in some breast intraductal carcinomas [12]. This analogy is interesting in that some features of breast pathology may reproduce events of breast development.

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