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VARIATION OF bcl-2 EXPRESSION IN BREAST DUCTS AND LOBULES IN RELATION TO PLASMA PROGESTERONE LEVELS: OVEREXPRESSION AND ABSENCE OF VARIATION IN FIBROADENOMAS

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SUMMARY

Some women with benign breast disease eventually develop breast cancer. The mammary gland undergoes tissue remodelling according to hormonal influences, involving a balance between quiescence, proliferation, and mechanisms of cell death. Proliferation and/or apoptotic events could therefore be investigated to help understand the mechanisms of benign lesion formation and identify mastopathies with a poor prognosis. bcl-2 expression was analysed by immunohistochemistry in 75 benign mastopathies. Protein levels were quantitated with an image analyser in various epithelial structures on frozen sections, including adenoses, fibroadenomas, ductal epithelial hyperplasias, cysts, and apparently normal surrounding lobules and ducts. bcl-2 levels were equivalent in apparently normal lobules and ducts, as well as in cysts and ductal hyperplasias. bcl-2 staining was significantly higher in fibroadenomas, known to be of lobular origin [mean = 10·1, quantitative immunochemistry score (QIC) arbitrary units (AU), $n = 19$], than in normal lobules (mean = 5·1 AU, $n = 43$, $P = 7 \times 10^{-5}$). bcl-2 levels in normal lobules and ducts varied according to the menstrual cycle, being higher during the follicular than the luteal phase ($P = 1{\cdot}8 \times 10^{-2}$ and $P = 1{\cdot}7 \times 10^{-2}$, respectively). This was further supported by a statistical link ($P = 5 \times 10^{-3}$) between high levels of circulating progesterone and weak bcl-2 staining in lobules and ducts. This progesterone-dependent variation was absent in fibroadenomas. No statistical correlation was found between bcl-2 expression and circulating levels of oestradiol, and follicle-stimulating or luteotrophic hormones. Although these are only preliminary results, they suggest an influence of progesterone on bcl-2 expression which might be lost in fibroadenomas. A hypothesis is proposed concerning the potential involvement of altered regulation of the apoptotic process in the formation of such benign lesions. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Mastopathies include a wide range of lesions, from 'subnormal' tissue to atypia.[1] Clarification of the different mechanisms leading to the appearance of such a variety of lesions could help to improve breast cancer risk prediction for women with mastopathies. Genetic alterations underlying breast cancer development vary according to tumour type[2] and it is likely that they are responsible for the degree of aggressiveness of the disease and consequently the outcome. Moreover, in benign lesions, gene alterations and/or deregulation leading to 'non-proliferating' mastopathies are probably not the same as those involved in the development of proliferating diseases or atypical hyperplasias. Under physiological conditions, the mammary gland undergoes cyclic tissue remodelling resulting from an alternation of quiescence, multiplication, and cell death, according to the extent of hormonal influence.[3–5] Our hypothesis concerning the development of various types of mastopathies is that deregulation of genes involved in the cell death process or in the cell cycle might lead to mastopathies with different behaviours.

The bcl-2 gene was first discovered in non-Hodgkin's B-cell lymphomas, where it is involved in the t(14–18) chromosomal translocation, leading to its transcriptional deregulation. bcl-2 was shown to extend cell life

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by preventing the onset of apoptosis under various conditions that induce cell death (for a review see ref. 6). In solid tumours, variable levels of bcl-2 protein have been described in the prostate,[7] nasopharynx,[8] lung,[9] colon,[10] and breast.[11–15]. In breast cancer, there is a close statistical correlation between high levels of bcl-2 protein and the presence of oestrogen receptor in tumour cells.[11,12,14,15] These observations, along with the fact that hormone-dependent breast tissue remodelling occurs, strongly suggest a link between hormones and bcl-2 expression in the breast. In the present study, we have quantified the expression of bcl-2 as revealed by immunohistochemistry in a series of 75 samples of benign breast disease with known plasma sex hormone levels.

MATERIALS AND METHODS

Patients and breast specimens

Benign mastopathy tissues were collected from 75 patients who underwent surgery for benign breast disease at the Val d'Aurelle-Paul Lamarque Cancer Centre in Montpellier (France). Patients with a personal history of breast cancer were not included in this study. One woman had a first-degree family history of breast cancer (mother and/or sister) and seven had a second-degree family history of breast cancer (aunt and/or grandmother). Specimens were snap-frozen in liquid nitrogen immediately upon arrival in the pathologist's laboratory, i.e., within 15 min after surgical removal at most. Samples were stored at -70°C until use. Histological examination of paraffin blocks led to the following major diagnoses: seven adenoses including four sclerosing adenoses, 31 fibroadenomas including 14 complex fibroadenomas, nine ductal hyperplasias without atypia, five ductal hyperplasias with atypia, and 23 cases of fibrocystic change.

Plasma sex hormone determination and patients' hormonal status

For all patients at surgery, a blood sample was systematically taken and the circulating hormone levels were determined on a routine basis. Plasma oestradiol and progesterone levels were measured using ESTR-CTRIA and the PROG-CTRIA kits, respectively (CIS Bio International, ORIS Group, Gif sur Yvette, France). Follicle-stimulating hormone (FSH) and luteotrophic hormone (LH) were detected with ^{125}I-hFSH and ^{125}I-hLH COATRIA kits, respectively (BioMérieux SA, Marcy-l'Etoile, France). Oestrogen and progesterone levels were given in pg/ml and ng/ml, respectively. FSH and LH were given in mUI/ml (conversion factor is 2nd IRP MRC 78/549). The hormonal status was determined according to hormonal levels[16] and to the date of the last menstruation when available. Plasma hormone levels were available for 72 patients out of 75. Our population included 60 premenopausal women with an age range of 18–53 (mean = 38) years, and 12 post-menopausal women with an age range of 47–82 (mean = 56) years. Three of the premenopausal women and three of the post-menopausal women were undergoing progesterone, local oestrogen, or oestroprogestative treatment, which was not reflected in their plasma sex hormone levels. The distribution of premenopausal patients according to the three phases of the menstrual cycle was as follows: follicular, 27; peri-ovulatory, 5; and luteal, 28.

Quantitative immunohistochemistry

bcl-2 protein was detected by immunohistochemistry using a mouse anti-human monoclonal antibody (clone 124, Dako A/S, Copenhagen, Denmark). A mouse anti-human IgG1 antibody was used as a negative control (Flobio, France). Cryosections of snap-frozen samples were laid on Superfrost/plus slides (Menzel-Glaser, U.S.A.), immediately fixed at -20°C in a 50:50 methanol–acetone solution for 10 min, and dried for 5 min at room temperature. Slides were then stored at -70°C until use. bcl-2 detection was carried out at room temperature in a Shandon apparatus using the alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase technique (APAAP, Dako A/S, Copenhagen, Denmark). Briefly, once rehydrated in 50 mM Tris (pH 7·6) and 150 mM NaCl, tissue sections were incubated for 30 min with a 1:50 dilution of the bcl-2 antibody. Following a 5 min rinse with 50 mM Tris (pH 7·6) and 150 mM NaCl, slides were incubated with an anti-mouse IgG (Dako A/S, Copenhagen, Denmark) and processed at pH 7·6 using the APAAP Dako kit. Detection was done at pH 8·2 with Fast Red chromogen (BioGenex, San Ramon, U.S.A.) mixed with 0·5 mM levamisole. Slides were finally counterstained with a 20 per cent haematoxylin solution and mounted for examination. bcl-2 staining was systematically quantified less than 1 week after the immunohistochemical reaction. Staining was quantified under a Leitz Diaplan microscope ($\times 25$ lens, Leica, Westlar, Germany) connected to a SAMBA 2001 image analyser using 'TEST IMMUNO' and 'IMMUNO' software packages (TITN, Grenoble, France). Cytoplasmic staining was quantified according to a previously described procedure.[17] Haematoxylin counterstaining was used to determine cellular areas and the background was evaluated on the corresponding negative control sections. The results of three experiments were processed independently for each tissue sample and bcl-2 staining was evaluated on every epithelial structure present on frozen sections.

As mastopathies are heterogeneous, in addition to the main lesion leading to the diagnosis, we also identified apparently normal structures and further lesions. Our series thus included the following epithelial structures: 12 adenoses, 19 fibroadenomas, 12 ductal hyperplasias, 9 cysts, 43 apparently normal lobules, and 42 apparently normal ducts. The 43 apparently normal lobules were removed from the sites of three adenoses, ten fibroadenomas, 11 ductal hyperplasias, and 19 examples of fibrocystic change. The 42 apparently normal ducts were removed from the sites of three adenoses, ten fibroadenomas, 11 ductal hyperplasias, and 18 examples of fibrocystic change. Quantitation was generally performed on five fields per structure analysed on

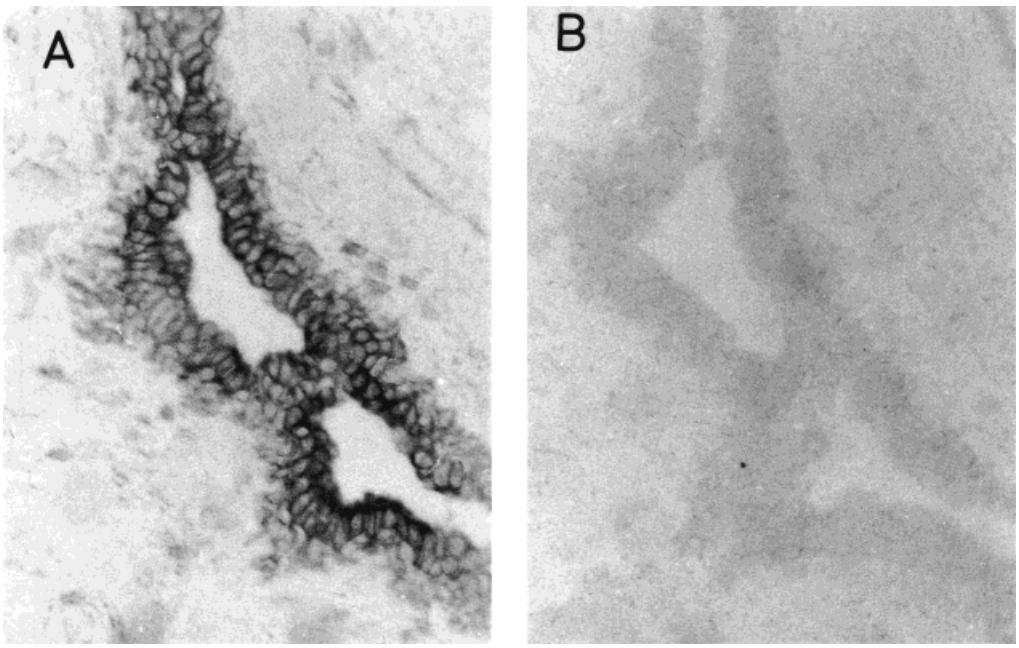

Fig. 1—bcl-2 protein immunostaining in epithelial cells of a fibroadenoma: (A) test and (B) control sections. Immunohistochemistry was performed on snap-frozen sections as described in the Materials and Methods section. Scale bar = 50 μm

each section. Results were expressed as a quantitative immunohistochemical (QIC) score, taking the staining intensity and the number of stained cells into account through the following formula: (labelling index) × (mean optical staining density)/100. The labelling index corresponded to the proportion of stained versus total cellular areas present in the field analysed. The mean optical density was determined on the basis of optical densities measured in the cytoplasm of labelled cells in the field. For each structure, the QIC score used for the statistical analysis was given by the mean of QIC score values measured on the three sections analysed.

Statistical analysis

Data were stored using the Paradox 4.5 database management system (Borland, Scotts Valley, CA, U.S.A.) in an IBM-compatible PC. Statistical analysis was performed with ANGOSS KNOWLEDGE SEEKER software (Angoss Software Intl Ltd., Toronto, Canada). Statistical differences were determined using chi-square and *F*-tests.[18,19] Clinical parameters used in this study were patient's age, hormonal status, family history of breast cancer, and hormonal treatments. Histological criteria were also included and our results were analysed as a function of the histological types of structures present on the immunostained sections. The presence of microcalcifications detected on paraffin sections was also considered.

RESULTS

bcl-2 expression in benign breast disease

Fixing tissues prior to paraffin embedding is a slow process that may affect gene expression. Due to the rapid renewal of proteins involved in the balance between cell cycling and cell death, we used snap-frozen samples to avoid bcl-2 degradation. To test the lability of this protein and assess the technical constraints necessary to preserve it in frozen tissue sections, we detected bcl-2 by immunohistochemistry on pieces of the same sample frozen at different times after surgical removal. Half of the bcl-2 signal was lost 30 min after the sample was removed from the patient and left at room temperature, and protein staining was totally absent in samples that were left for 45 min (data not shown). bcl-2 protein staining was cytoplasmic, as previously described.[11–15] Although bcl-2 staining was noted in myoepithelial and stromal cells of some samples, it was mainly present and evenly distributed in epithelial components of the 75 mastopathies studied (Fig. 1). Staining was thus quantified in the different epithelial structures of each section and mean levels were calculated from the results of three experiments per patient. bcl-2 levels were graded in QIC score arbitrary units (AU) based on cellular staining and ranged from 0·6 to 24·8 AU depending on the samples studied.

Correlation between bcl-2 expression and histopathological parameters

We looked for statistical associations between bcl-2 expression levels and clinicopathological parameters. We did not find any correlation with the presence of microcalcifications in the samples. According to the histological examination of frozen sections used for bcl-2 detection, our series of samples contained the following structures: apparently normal lobules and ducts, adenoses, fibroadenomas, ductal hyperplasias, and cysts. bcl-2 staining in apparently normal lobules and ducts was the same, regardless of the accompanying

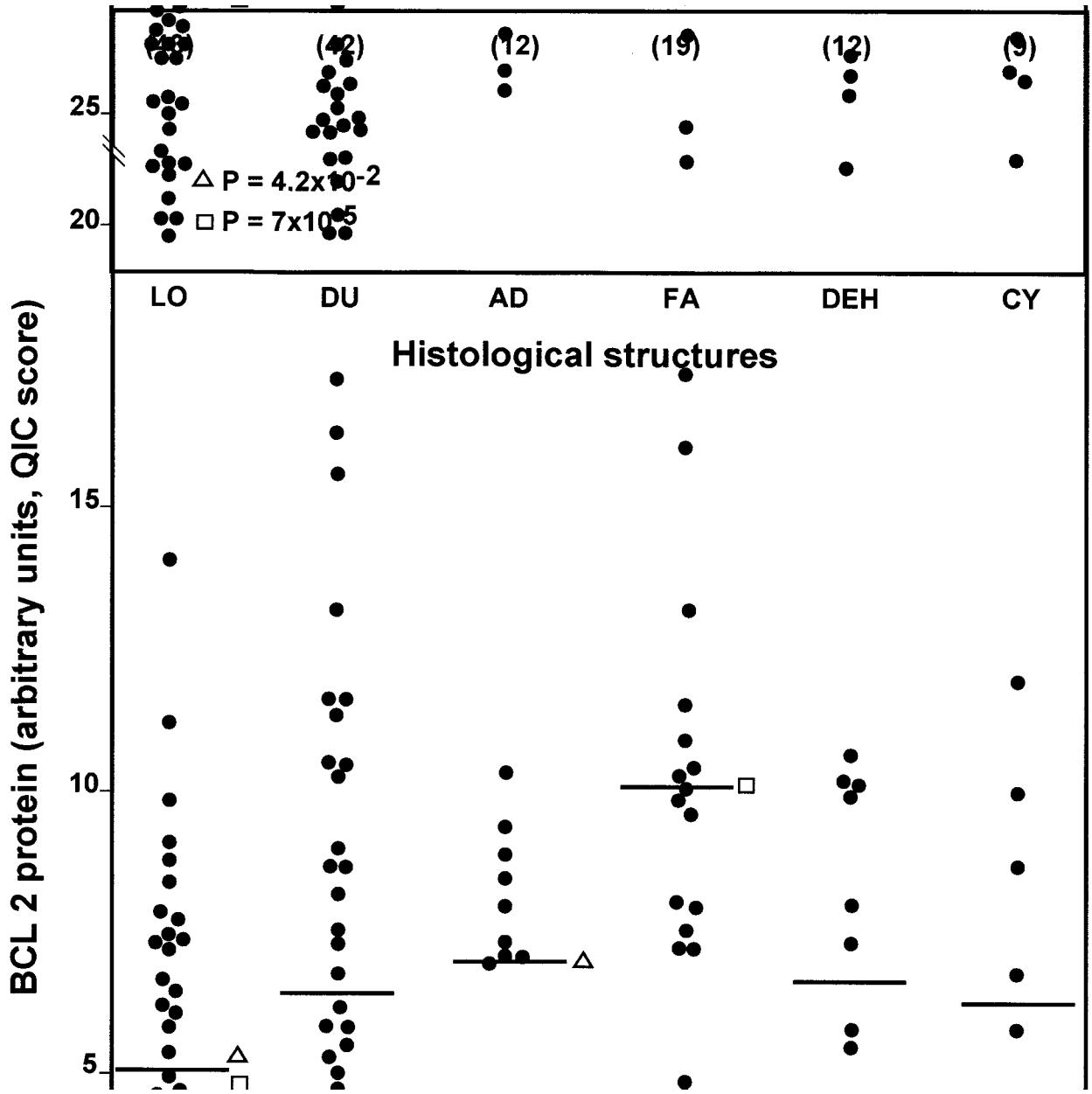

Fig. 2—Levels of bcl-2 protein immunostaining quantified in various epithelial histological structures present on frozen sections of benign mastopathies: LO, lobules; DU, ducts; AD, adenoses; FA, fibroadenomas; DEH, ductal epithelial hyperplasias; CY, cysts. bcl-2 levels are given as means of three independent experiments for each patient. Quantitative immunohistochemistry (QIC) was performed as described in the Materials and Methods section and the results are given in QIC score arbitrary units. Numbers in parentheses correspond to the number of mastopathies and horizontal bars represent means. bcl-2 levels observed in each mastopathic structure were statistically compared with bcl-2 levels in the corresponding apparently normal structure: adenoses or fibroadenomas versus lobules and ductal hyperplasias versus ducts. *P* values were determined by the *F*-test. Significant correlations were observed when comparing bcl-2 levels in apparently normal lobules with adenoses (△) or fibroadenomas (□)

Table I—bcl-2 expression according to patients' hormonal status

Epithelial structure	Menstrual cycle phase			*P* value	Menopause
	Follicular	Peri-ovulatory	Luteal	Follicular/luteal	
Lobules	6·06 ($n=14$)	3·88 ($n=5$)	3·34 ($n=14$)	0·017	7·33 ($n=7$)
Ducts	8·07 ($n=13$)	5·64 ($n=3$)	3·77 ($n=14$)	0·018	7·97 ($n=9$)
Adenoses	8·25 ($n=4$)	—	6·22 ($n=7$)	NS	7·97 ($n=1$)
Fibroadenomas	10·9 ($n=7$)	—	9·08 ($n=11$)	NS	16·04 ($n=1$)
Ductal hyperplasias	9·19 ($n=3$)	5·51 ($n=1$)	4·67 ($n=6$)	0·04	9·32 ($n=2$)
Cysts	8·71 ($n=3$)	4·32 ($n=2$)	5·23 ($n=3$)	—	5·80 ($n=1$)

bcl-2 expression was measured by quantitative immunohistochemistry (QIC) on epithelial structures present on frozen sections. bcl-2 levels are given in QIC score arbitrary units as described in the Materials and Methods section. The patients' hormonal status was determined according to the levels of their plasma sex hormones at surgery[16] and the date of the last menstruation when available. The menstrual cycle was subdivided into follicular, peri-ovulatory, and luteal phases. Numbers in parentheses indicate the number of patients in each category; *P* values were determined by the *F*-test; NS = non-significant.

pathology. We then compared bcl-2 levels in the different lesions with those observed in apparently normal histological structures. As shown in Fig. 2, bcl-2 staining intensity in apparently normal lobules ranged from 0·6 to 14·2 AU, with a mean of 5·1 ($n=43$). bcl-2 levels in apparently normal ducts were not statistically different (range 0·7–17·4 AU, mean = 6·4, $n=42$). In cysts, mean expression levels were equivalent to those observed in apparently normal lobules and ducts (mean = 6·2 AU, $n=9$). We further compared bcl-2 expression in various lesions with that measured in the apparently normal structure from which they originated. bcl-2 expression in ductal epithelial hyperplasia (mean = 6·6 AU, $n=12$) was equivalent to that observed in apparently normal ducts. Since fibroadenomas and adenoses are supposed to originate from lobules,[20] bcl-2 expression in these lesions was compared with that observed in apparently normal lobules. The statistical analysis showed that levels of bcl-2 increased significantly in fibroadenomas (mean = 10·1 AU, $n=19$) compared with lobular levels ($P=7\times10^{-5}$), and increased to a lesser extent in adenoses (mean = 7 AU, $n=12$) ($P=4{\cdot}2\times10^{-2}$). Moreover, when comparing bcl-2 levels in fibroadenomas with those in apparently normal lobules derived exclusively from fibroadenomatous samples, bcl-2 levels were significantly higher ($P=1{\cdot}2\times10^{-2}$) in fibroadenomatous structures (mean = 10·1 AU, $n=19$) than in surrounding lobules (mean = 5·39 AU, $n=10$). There was no atypical hyperplasia in our series of frozen sections. No increased expression was observed in apparently normal ducts and lobules, or in ductal hyperplasia from samples with atypical hyperplasia.

Correlation between sex hormones and bcl-2 expression

Since the mammary gland undergoes remodelling during the menstrual cycle,[3] we looked at bcl-2 expression according to patients' hormonal status. This status was determined as a function of the patients' age and levels of circulating sex hormones (FSH, LH, oestrogen, and progesterone).[16] As shown in Table I, bcl-2 levels in lobules and ducts varied according to the menstrual cycle, being higher during the follicular than the luteal phase ($P=1{\cdot}7\times10^{-2}$ and $1{\cdot}8\times10^{-2}$, respectively). In contrast, fibroadenomas had the same bcl-2 levels during the follicular and luteal phases of the menstrual cycle. Adenoses, ductal hyperplasias, and cyst populations were too small to draw any valid conclusions.

In an attempt to define further which sex hormone(s) could be involved in bcl-2 regulation, we looked for statistical correlations between bcl-2 expression in the different epithelial structures and the levels of all circulating hormones. We did not find any correlations between FSH, LH or E_2 levels and bcl-2 protein staining in any epithelial structure analysed. A plasma progesterone level of 1 ng/ml was used as a cut-off point for comparisons, to investigate a possible correlation between progesterone and bcl-2 staining. This threshold was chosen since levels above 1 ng/ml reflect luteal activity. An inverse statistical correlation was found between progesterone levels and bcl-2 expression in apparently normal lobules and ducts (Fig. 3). Accordingly, high mean levels of bcl-2 (lobules: mean = 6·2 AU; ducts: mean = 7·8 AU) were accompanied by low plasma progesterone levels (<1 ng/ml), whereas lower bcl-2 levels (lobules: mean = 3·5 AU; ducts: mean = 3·9 AU) corresponded to higher progesterone levels ($\geqslant$1 ng/ml) (both $P=5\times10^{-3}$). In fibroadenomas, bcl-2 levels were not statistically different ($P=0{\cdot}5$), regardless of the progesterone (Pg) level (Pg<1 ng/ml, mean bcl-2 = 11·5 AU; Pg$\geqslant$1 ng/ml, mean bcl-2 = 9·1 AU). Although there were not enough examples of the other histological structures to draw definite conclusions, this variation with Pg levels was also observed in ductal hyperplasias, but not in adenoses or cysts.

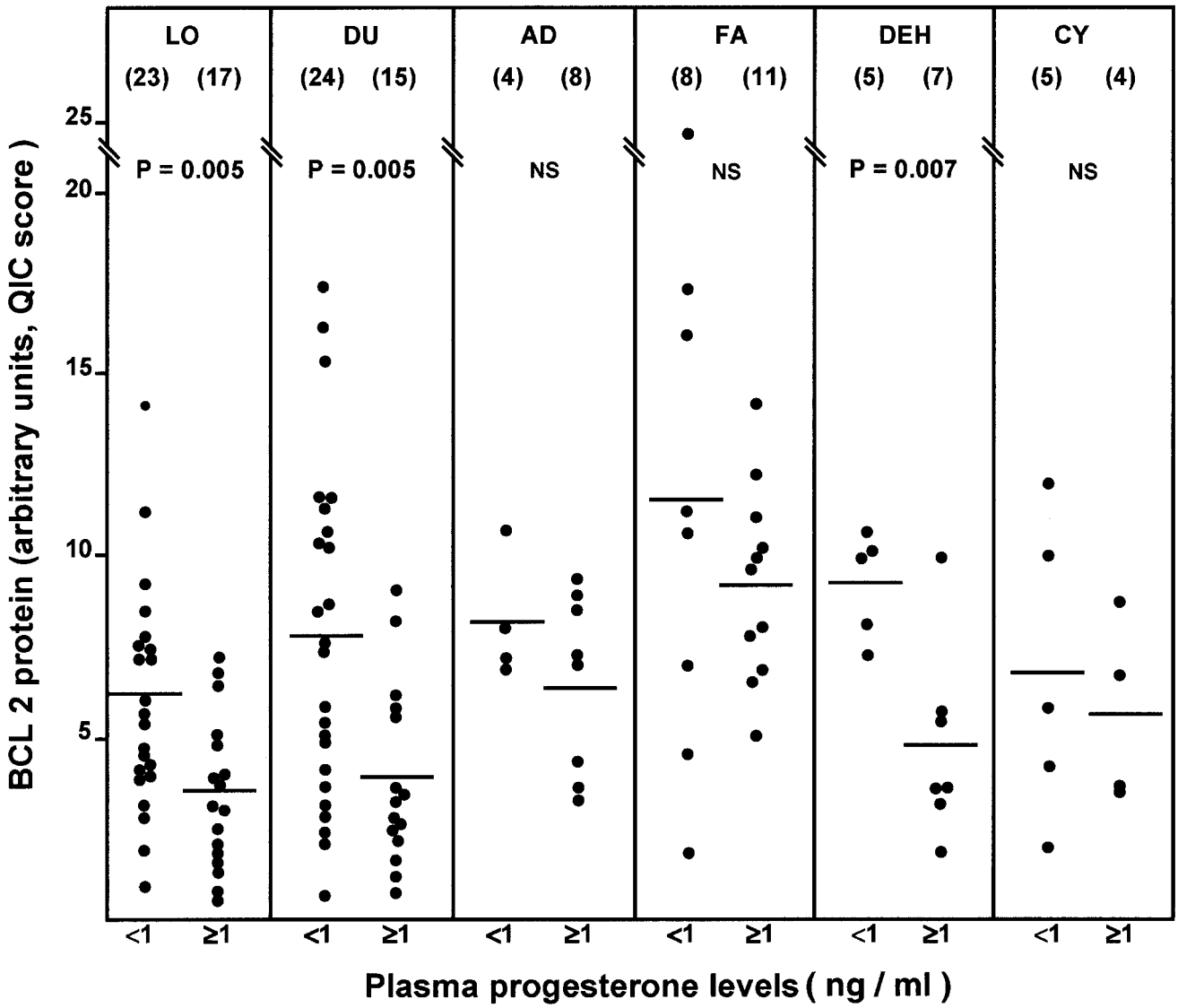

Fig. 3—Variations in bcl-2 protein immunostaining levels according to plasma progesterone. bcl-2 levels were quantified in various epithelial histological structures present on frozen sections of benign mastopathies: LO, lobules; DU, ducts; AD, adenoses; FA, fibroadenomas; DEH, ductal epithelial hyperplasias; CY, cysts. bcl-2 levels are given as means of three independent experiments for each patient. Quantitative immunohistochemistry (QIC) was performed as described in the Materials and Methods section and results are given in QIC score arbitrary units. Numbers in parentheses correspond to the number of mastopathies and horizontal bars represent means. In each category of histological structure, variations in bcl-2 immunostaining were statistically evaluated according to plasma progesterone levels; *P* values were determined by the *F*-test

Elevated bcl-2 levels in fibroadenomatous structures in the presence of high progesterone levels could be explained by the absence of progesterone receptor in epithelial cells of fibroadenomas. We thus carried out the immunohistochemical detection of the progesterone receptor. All samples with progesterone levels above 1 ng/ml had progesterone receptors in their epithelial cells (data not shown). This indicates that there might have been either deregulation of bcl-2 expression or progesterone receptor malfunction. The loss of biological activity of the receptor could thus prevent down-regulation of bcl-2 by progesterone. Further studies would be necessary to answer this question.

Overall, these results suggest a potential loss of bcl-2 control in fibroadenomas, with increased expression regardless of progesterone levels. Fibroadenomas were recently subdivided into two categories according to their histological features.[21] Fibroadenomas containing additional lesions considered as 'complex fibroadenomas' would be at higher risk of breast cancer than non-complex ones. In our study, bcl-2 levels were the same in both fibroadenoma groups, indicating that the presence of additional lesions surrounding fibroadenomas did not interfere with their bcl-2 levels.

DISCUSSION

We measured bcl-2 protein levels in frozen sections of breast tissues from patients who underwent surgery for benign breast disease. bcl-2 protein levels were determined in every histological structure present on tissue

sections, including lesions and apparently normal lobules and ducts. In some cases, however, we received only normal tissue. In our population, we detected similar mean levels of bcl-2 in apparently normal lobular and ductal structures. This is in contrast with the study of Sabourin *et al.*,[22] who found bcl-2 preferentially expressed in lobules. This discrepancy may be explained by the fact that these authors used paraffin-embedded samples, which may not be adequate for preserving highly unstable proteins such as bcl-2 (see Results), a preliminary observation which prompted us to work with frozen sections. During fixation at room temperature prior to paraffin embedding, the bcl-2 protein could thus be more stable in lobules than in ducts. In addition to apparently normal lobules and ducts, our series of frozen tissue sections included adenoses, fibroadenomas, ductal hyperplasias, and cysts. bcl-2 was detected in all types of structures and levels were significantly increased in fibroadenomas ($P = 7 \times 10^{-5}$) and, to a lesser extent, in adenoses ($P = 4{\cdot}2 \times 10^{-2}$), compared with levels in lobules. This suggests a specific change in the control of bcl-2 expression in these lesions. Our results concerning fibroadenomas were in agreement with the decreased apoptosis to mitosis ratio in these lesions observed by Allan *et al.*[23] Conversely, levels in ductal hyperplasia were equivalent to those observed in apparently normal ducts.

c-myc gene expression is modulated according to the menstrual phase in mastopathies,[24] indicating that genes possibly involved in mammary tumourigenesis can be under the influence of sex hormones *in vivo*. Moreover, as bcl-2 overexpression is associated with the presence of steroid receptors in breast cancer, we compared our results with the patients' hormonal status and found that, like c-myc, bcl-2 expression was high during the follicular phase and diminished throughout the menstrual cycle in apparently normal lobules and ducts removed from the site of benign lesions. More precisely, this diminution of bcl-2 levels was correlated with elevated plasma progesterone levels ($\geqslant 1$ ng/ml). Although the pattern of bcl-2 expression differed from that observed by Sabourin *et al.* in the first part of the menstrual cycle,[22] both their results and ours on the post-ovulatory period matched the description of tissue remodelling manifested by apoptosis at the end of the menstrual cycle in the absence of fertilization.[3] There was no correlation between bcl-2 and progesterone in fibroadenomas, since bcl-2 levels remained high in these benign mastopathies, regardless of the menstrual cycle. These results suggest a loss in the potential control of bcl-2 by progesterone in such diseases originating from epithelial lobular components. There was no correlation with oestrogen levels in any of the structures analysed. This is in contrast with the *in vitro* modulation of bcl-2 expression according to oestrogen levels observed in the MCF7 breast cancer cell line.[25] Moreover, in breast cancer biopsies, a correlation has been found between the presence of ER and high bcl-2 levels.[11,12,14,15] Although this study should be performed on a larger number of samples, these preliminary results suggest that bcl-2 dysregulation might more specifically occur in benign lesions derived from lobules.

In benign breast diseases, adenoses and fibroadenomas are lesions whose risk of further development into breast cancer has been described as equivalent to[26,27] or slightly higher than[28–32] that of women without breast disease in the general population. In addition to this observation, our data suggest that fibroadenomas might result from a slow accumulation of cells, because the apoptotic step is skipped during menstrual cycles. Apoptosis and proliferation are successive events during the menstrual cycle.[3] A defect in the apoptotic process, along with a physiological proliferation rate, might prompt an accumulation of cells. This physiological proliferation rate would thus lead to the same proportion of genetic alterations as that of the general population. Women bearing these lesions with 'apoptosis deficient cells' would then not present a much higher risk of breast cancer than women without breast disease. However, if a mutation occurs during cyclical proliferation, the failure to eliminate newly mutated cells would then increase the risk of developing breast cancer. Our hypothesis is that additional events, perhaps in the proliferation process, might be necessary to increase further the risk of breast cancer. Additional studies on a large number of mastopathies, investigating a variety of genes involved in cell cycling and/or apoptotic processes, will be necessary to confirm this hypothesis.

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