Apoptosis during Breast Carcinoma Progression[1]

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ABSTRACT

The purpose of this study was to investigate apoptosis, proliferation, and the expression of apoptosis-influencing proteins bcl-2 and bax and estrogen and progesterone receptors during breast carcinoma progression. The material consisted of 53 paired breast carcinoma samples representing primary and recurrent tumors and 24 control samples. The recurrent sample was located either in the breast scar tissue or at a distant metastatic site. Apoptosis was detected both morphologically and by 3′ end labeling of fragmented DNA. Cell proliferation was evaluated immunohistochemically by the MIB index. The expressions of bcl-2, bax, and estrogen and progesterone receptors were studied immunohistochemically. There was a significant increase in the extent of apoptosis and proliferation in recurrent tumors compared to the primary lesions ($P = 0.015$ and $P = 0.038$, respectively). In primary tumors with an apoptotic index of >0.50%, the survival of the patients was significantly shorter ($P = 0.015$). In cases with a significant increase in apoptosis or proliferation in the recurrent tumor, the survival of the patients was significantly shorter ($P = 0.009$ and $P = 0.003$, respectively). Of the variables analyzed, bcl-2 expression and a positive estrogen receptor status were significantly associated with a low extent of apoptosis ($P = 0.010$ and $P = 0.042$, respectively). Their changes were parallel to the changes in apoptosis during tumor progression, although the associations did not reach statistical significance. The results show that increased apoptosis is associated with a worse prognosis in breast carcinoma. A significant increase in apoptosis in recurrent breast carcinoma lesions predicts a worse clinical outcome.

INTRODUCTION

Apoptotis is an actively regulated cellular process that leads to the destruction of individual cells (1–4). It can be triggered by several stimuli, such as radiation, drugs, and toxins, or by deprivation of hormones or growth factors (5, 6). Morphologically, apoptotic cells are characterized by nuclear shrinkage and pyknosis, eventually leading to nuclear fragmentation and phagocytosis of the apoptotic cells (3, 7). Biochemically, the end result of an apoptotic process is DNA fragmentation by endonucleases to periodic 180-bp fragments, which can be detected in DNA electrophoresis as a typical ladder pattern (3). The DNA fragmentation and nuclear destruction are regulated by caspases, which are proteolytic enzymes capable of cleaving amino acids at aspartatic acid-alanine residues (8, 9).

The importance of apoptosis in tissue homeostasis is reflected by the fact that it is influenced by several cancer genes, such as *p53* and *Rb* tumor suppressor genes and c-*myc* (4, 5). An important group of genes influencing apoptosis is the *bcl-2* family (1–7). The proteins encoded by these genes can either promote or inhibit apoptosis (1–7). bcl-2, for instance, is able to inhibit apoptosis (10). Translocation of the *bcl-2* gene to an active promoter site has been shown to be an important genetic change in the development of follicular lymphomas (11, 12). Other members of the bcl-2-related group include bax, bad, bcl-xL, bcl-xS, mcl-1, and so on (1–5). These proteins may either homodimerize or form heterodimers with each other (1–5). The dimerization is important for the apoptosis regulating function of these proteins (1–5). An important regulatory factor for apoptotic regulation in many cells is the balance between the concentrations of bax and bcl-2 proteins. In cases in which bax is in excess, bax-bax homodimers, which lead to a promotion of apoptosis, predominate (4, 5). bcl-2, however, may heterodimerize with bax, and its increased concentration leads to formation of bcl-2-bax heterodimers, which favor abrogation of apoptosis (4, 5). Other members of the bcl-2 group may also heterodimerize with bax (4, 5). bcl-xL, for instance, by forming heterodimers with bax, is also able to inhibit apoptosis (5). bad, on the other hand, binds bcl-2 and bcl-xL, leading to an excess of bax homodimers and a promotion of apoptosis (5). Many members of the bcl-2 family are resident proteins of the mitochondrial membrane, and they have been shown to influence the mitochondrial membrane potential or to promote or inhibit the release of caspase-activating substances such as cytochrome *c* or apoptosis-inducing factor from mitochondria (13, 14). In line with their antiapoptotic function, bcl-2 and bcl-xL inhibit the release of cytochrome *c* or apoptosis-inducing factor from mitochondria, whereas the proapoptotic bax promotes it (13, 14).

Apoptosis is often increased in malignant tumors (15, 16). In breast carcinoma, increased apoptosis is associated with a negative estrogen or progesterone receptor status, a low degree of differentiation, tumor aneuploidy, and a decreased bcl-2 expression (17). Bcl-2 expression is present in ~70% of breast carcinomas (18, 19). Strong bcl-2 expression is associated with a positive estrogen or progesterone receptor status (20, 21). Its expression is related to a favorable prognosis (19, 20) but is not an independent prognostic factor (19). In node-positive breast carcinomas, however, bcl-2 expression is independently related to a better prognosis (18, 20).

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The aim of this study was to investigate apoptosis in a set of breast carcinomas from which samples of tumors could be obtained from at least two temporally different occasions. As a control group, we included 12 paired cases, in which samples were obtained from two histologically different tumors from different breasts of the same patients. The sections were also studied immunohistochemically for cell proliferation using the MIB-1 antibody and for the expression of bcl-2, bax, and estrogen and progesterone receptors to see whether changes in apoptosis could be ascribed to putative changes in their expression. The results were correlated with clinical data, such as survival of the patients.

MATERIALS AND METHODS

Materials. Fifty-three samples of breast carcinoma consisting of the primary and recurrent lesion and a control group of 12 cases representing two histologically different carcinomas from different breasts of the same patients were collected from the files of the Department of Pathology, University of Oulu, between the years 1979 and 1996. The material with the primary and recurrent samples consisted of 44 ductal carcinomas, 8 lobular carcinomas, and 1 mucinous carcinoma. In the control group there were 21 ductal carcinomas, 2 lobular carcinomas, and 1 apocrine carcinoma. The diagnosis was based on the WHO classification of breast tumors (22). The grades of the ductal carcinomas were evaluated according to Bloom and Richardson (23). All but seven tumors represented cases with nodal axillary metastases at the primary occasion. The total follow-up time between the primary and recurrent tumor in the paired sample group was 148.7 years (mean = 2.8 years, range = 0.3–10.9 years). In all but three cases, a full mastectomy was performed as an initial treatment. Forty patients received radiation therapy, 25 received antiestrogen (tamoxifen), and 11 received cytostatic therapy during the follow-up.

3′ End Labeling of DNA in Apoptotic Cells. To detect apoptotic cells, *in situ* labeling of the 3′ ends of the DNA fragments generated by apoptosis-associated endonucleases was used. The 3′ end labeling of DNA was performed using the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD), as described previously (15, 16). A positive control consisted of a lymph node with follicular hyperplasia.

Cells were defined as apoptotic if their whole nuclear area labeled positively. Apoptotic bodies were defined as small positively labeled globular bodies in the cytoplasm of the tumor cells that could be found either singly or in groups. To estimate the apoptotic index, the numbers of apoptotic cells and bodies were counted in 10 HPFs[3] with a total area of $\sim$1.2 mm^2 (10 HPFs, $\times$40 objective, diameter of the field = 400 μm) and divided by the total number of tumor cells in this area. Cells in the vicinity of necrotic areas were not assessed.

In addition to the 3′ end labeling method, we also performed apoptosis assessment by light microscopy based on the morphological criteria of apoptosis (3). The morphological apoptosis was assessed from the same tumor samples, and the estimation of the apoptotic index was performed in a manner similar to that of the 3′ end labeling method.

Immunohistochemical Staining. Five-μm sections were cut from the specimens, dewaxed in xylene, and rehydrated in graded alcohol. The endogenous peroxidase was consumed by immersing the sections in 0.1% hydrogen peroxide in absolute methanol for 20 min. Nonspecific binding was blocked by incubating the slides in 20% FCS in PBS for 20 min.

For MIB-1 staining, a dilution of 1:25 was used for the primary monoclonal antibody (Immunotech, Marseilles, France). This was followed by a secondary biotinylated rabbit antimouse antibody (dilution = 1:300; Dakopatts, Copenhagen, Denmark) and the avidin-biotin-peroxidase complex (Dakopatts).

A monoclonal antibody (clone 124) against bcl-2 oncoprotein was obtained from DAKO (Glostrup, Denmark). A polyclonal antibody to bax was obtained from PharMingen (San Diego, CA). Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mM citric acid monohydrate (pH 6.0) for 5 min. After a 30-min incubation with the primary antibody (dilution = 1:50 for bcl-2 and 1:1000 for bax), a biotinylated secondary antimouse or antirabbit antibody (both from Dakopatts) was applied (dilution 1:300) followed by the avidin-biotin-peroxidase complex (Dakopatts).

For all of the immunostainings, the color was developed by diaminobenzidine, after which the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Negative control stainings were carried out by substituting nonimmune mouse or rabbit serum for the primary antibodies. A positive control consisted of a lymph node with follicular hyperplasia.

The proliferative activity, expressed as the MIB index, was determined as the number of MIB-positive cells divided by the sum of all tumor cells in 10 HPF areas studied. The intensity of the bcl-2 and bax immunostaining was evaluated by dividing the cytoplasmic staining reactions in four score groups: 1 = weak cytoplasmic staining intensity; 2 = moderate cytoplasmic staining intensity; 3 = strong cytoplasmic staining intensity; and 4 = very strong cytoplasmic staining intensity. The immunostaining was quantified as follows: 0 = no positive immunostaining; 1 = $<$25% of tumor cells showing cytoplasmic positivity; 2 = 25–50% of tumor cells showing cytoplasmic positivity; 3 = 50–75% of tumor cells showing cytoplasmic positivity; and 4 = $>$75% of tumor cells showing cytoplasmic positivity. A combined score for bcl-2 and bax immunostaining, based on both qualitative and quantitative analysis, was obtained by adding the qualitative and quantitative scores; these sums were then divided in three main groups: score = 0, no immunoreactivity; score = 1–4, weak immunoreactivity; and score = 5–8, strong immunoreactivity.

For estrogen and progesterone receptor staining, the slides were rehydrated and then microwaved in EDTA buffer first 3 min at 99°C and then 27 min in 85°C. The endogenous peroxidase was blocked with 0.1% hydrogen peroxide in absolute methanol for 20 min. Nonspecific staining was blocked by incubating the slides in normal FCS for 20 min, followed by the primary antibody and the avidin-biotin-peroxidase complex. The slides were counterstained with methyl green, and the

[3] The abbreviation used is: HPF, high-power field.

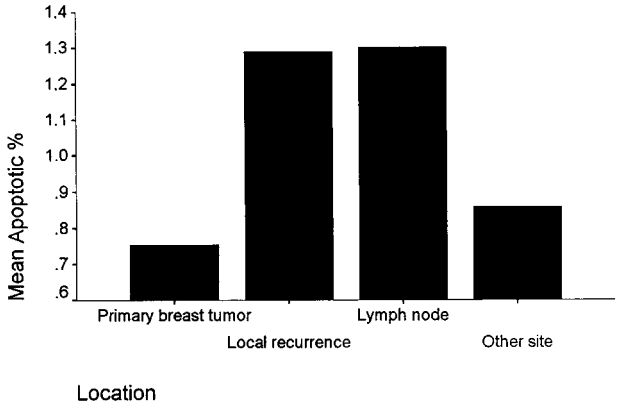

Fig. 1 The mean apoptotic index in the primary tumor and in recurrent tumors in different locations. The apoptotic index is clearly higher in recurrent tumors, but there is no significant difference between local recurrences and recurrences in metastatic sites.

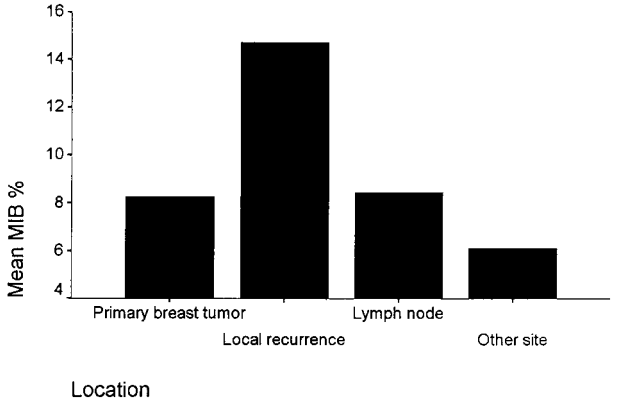

Fig. 2 The mean MIB index in the primary tumor and in recurrent tumors in different locations. The MIB index is clearly higher in local recurrences than it is in recurrences in metastatic sites or the primary tumor.

estrogen and progesterone receptor status was determined as described previously (24).

Statistical Analysis. SPSS for Windows (Chicago, IL) was used for statistical analysis. The significance of the associations were determined using the χ^2 test, Fisher's exact probability test, correlation analysis, and two-tailed *t* test. Univariate and multivariate analyses of survival data were undertaken by using survival curves and applying the Kaplan-Meier method with log-rank analysis and the Cox regression model. Probabilities of $P \leq 0.05$ were regarded as statistically significant.

RESULTS

Apoptotic and MIB Indices in Primary and Recurrent Breast Carcinomas. The average apoptotic index in all samples was 0.97 ± 1.27% (mean ± SD; range = 0.00–5.85%), and the MIB index was 9.26 ± 10.92% (range = 0.09–70.68%). There was a positive correlation between the apoptotic and MIB index ($r = 0.3997$, $P < 0.001$).

The average apoptotic index in the primary carcinoma lesions was 0.74 ± 1.02% (range = 0.00–5.85%), whereas in the recurrent tumors, it was 1.36 ± 1.42% (range = 0.00–5.77%; $P = 0.015$). In the 12 control cases from patients with two histologically different breast tumors, the mean apoptotic index did not show a statistically significant difference between the tumors (0.35 ± 0.56% and 0.18 ± 0.13%, respectively, $P = 0.41$).

The average MIB index in the primary breast carcinomas was 7.17 ± 7.55% (range = 0.14–51.86%), and in the recurrent carcinomas, it was 11.41 ± 13.27% (range = 0.32–70.45%; $P = 0.038$). In the control cases, no significant difference was observed (5.76 ± 5.74% and 5.74 ± 5.25%, respectively; $P = 0.85$).

Of the recurrent tumors, 16 were local recurrences, 23 were lymph node metastases of axillary or other sites, and the rest were metastases in other locations (skin, peritoneum, gastrointestinal tract, and liver). The apoptotic index was highest in recurrences in lymph nodes (1.35 ± 1.45%; $P = 0.07$ compared to the primary tumor) and in local recurrences in the breast tissue scar (1.31 ± 1.59%; $P = 0.10$), but it was somewhat lower in recurrences at other sites (0.95 ± 1.33%; $P = 0.51$; Fig. 1).

The MIB index was highest in local recurrences (18.72 ± 8.37%), as compared to the primary tumor ($P = 0.001$; Fig. 2). The MIB index in lymph nodes or other sites did not significantly differ from the primary tumor ($P = 0.50$ and 0.26, respectively; Fig. 2).

Patients with a primary tumor showing an apoptotic index of >0.50% had a significantly shorter survival ($P = 0.015$, log-rank; Fig. 3). Although patients with a high MIB index (>7.18%) in the primary tumor had a slightly worse prognosis, the difference was not statistically significant ($P = 0.50$, log-rank). Patients showing a >0.50% increase in apoptosis or a >2.00% proliferation per year had a significantly shorter survival than other cases ($P = 0.009$ and 0.0027, respectively; Figs. 4 and 5).

bcl-2, bax, and Estrogen and Progesterone Receptors in Primary and Recurrent Breast Tumors. A decrease in bcl-2 and bax immunoreactivity in recurrent *versus* primary tumors sample could be seen in 4 and 3 cases, respectively, and an increase was seen in 10 and 11 cases. A loss of a positive estrogen or progesterone receptor status could be seen in 6 and 2 cases, respectively. In cases with an increased bcl-2 immunoreactivity in the recurrent tumor, there was an average increase of only 0.15 ± 0.46% in the apoptotic index while in cases with no change or a decrease it was 0.71 ± 1.62% ($P = 0.10$). An increase in bax expression was not significantly associated with changes in apoptosis in the recurrent tumor (0.62 *versus* 0.56%, $P = 0.57$). In cases with a loss of positive estrogen or progesterone receptor status, there was an average increase of 1.39 ± 2.59% in apoptosis, whereas in the other cases, it was only 0.41 ± 1.29% ($P = 0.17$). Cases with a loss in the positive estrogen or progesterone status showed a significant increase in proliferation ($P = 0.029$). Changes in bcl-2 or bax expression did not significantly affect proliferation (data not shown).

Associations between bcl-2 and bax expression and estrogen and progesterone receptor status in primary and recurrent tumors are given in Table 1. There was no significant associa-

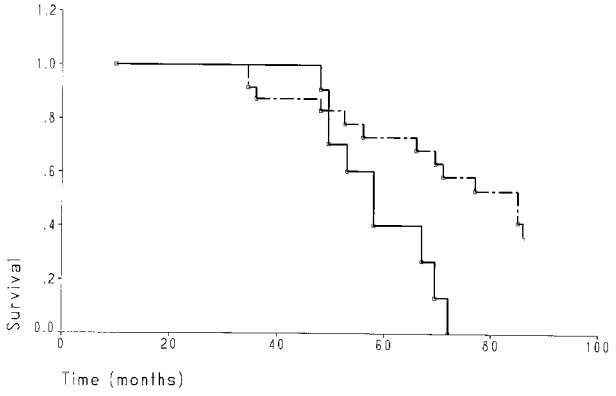

Fig. 3 Apoptosis and survival in primary breast carcinoma. The cumulative survival of patients with primary tumors showing an apoptotic index of >0.50% is significantly shorter than that of the other patients ($P = 0.015$, log-rank). ——, apoptosis > 0.50%; — - —, apoptosis ≤ 0.50%.

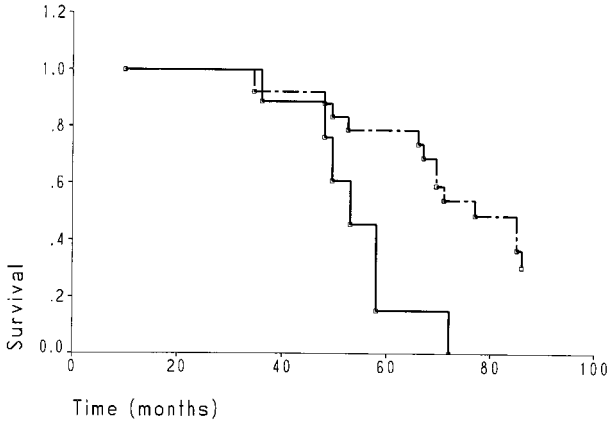

Fig. 4 Increased apoptosis rate and survival. In cases in which the apoptotic index in the recurrent tumor had increased >0.50% per year compared to the primary tumor, the survival of the patient was significantly shorter ($P = 0.009$, log-rank). Increased apoptosis: ——, change > 0.50%; — - —, change ≤ 0.50%.

tion between bcl-2 and bax expression ($P = 0.09$). Cases with no bcl-2 expression had a significantly higher apoptotic index than did cases that were positive for bcl-2. The apoptotic index in cases with no bcl-2 expression was 1.32 ± 1.43% (range = 0.00–5.85%), whereas in bcl-2-positive cases, it was 0.71 ± 1.10% (range = 0.00–5.77%; $P = 0.010$). Cases with a negative estrogen receptor status had an apoptotic index of 1.14 ± 1.50% (range = 0.00–5.85%), whereas receptor positive cases had an apoptotic index of 0.68 ± 0.87% (range = 0.00–3.78%; $P = 0.042$). Cases with a negative progesterone receptor status had a mean apoptotic index of 1.00 ± 1.04% (range = 0.00–5.85%), whereas for those with a progesterone positive status, it was 0.66 ± 0.97% (range = 0.00–3.18%; $P = 0.160$).

Patients with tumors showing a strong bcl-2 positivity (in the primary lesion) or a positive estrogen or progesterone receptor status had a better prognosis ($P = 0.02$, $P = 0.004$, and

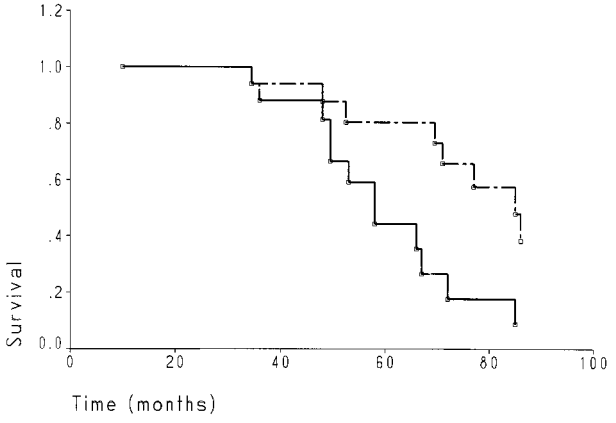

Fig. 5 Increase proliferation rate and survival. In cases in which the MIB index in the recurrent tumor had increased >2.00% per year compared to the primary tumor, the survival of the patient was significantly shorter ($P = 0.0027$, log-rank). Proliferation change: ——, change > 2.00%; — - —, change ≤ 2.00%.

$P = 0.01$, respectively, log-rank). The bax immunoreactivity, on the other hand, did not affect the survival ($P = 0.86$, log-rank).

Other Variables. Antiestrogen and cytostatic treatment did not significantly affect the rate of apoptosis or tumor cell proliferation in the recurrent tumors [$P = 0.46$ and $P = 0.97$ (antiestrogen treatment) and $P = 0.37$ and $P = 0.13$ (cytostatic treatment)]. Of 25 cases receiving antiestrogen therapy, 11 showed an apoptotic index of <0.50%. The corresponding figures for cases receiving radiation therapy and cytostatic treatment during the follow-up were 18 of 40 and 5 of 11, respectively. By Cox multivariate regression model, enhanced apoptosis showed a 2.0-fold risk for a shortened survival ($P = 0.016$, 95% confidence interval = 1.14–3.49) and appeared to be an independent prognostic variable, whereas sytostatic treatment ($P = 0.10$), tamoxifen ($P = 0.72$), and radiation therapy ($P = 0.99$) did not show such an association.

Comparison of the Morphological Apoptotic Index with the 3′ End Labeling Method. The average morphological apoptotic index was 0.87 ± 1.01% (range = 0.01–4.95%), giving a slightly lower index than 3′ end labeling. There was a statistically significant positive correlation between the morphological apoptotic index and the 3′ end labeling method ($r = 0.4498$, $P < 0.001$). The morphological apoptotic index was clearly lower in primary (0.69 ± 0.62%, range = 0.01–2.98%) than in recurrent (1.14 ± 1.30%, range = 0.01–4.96%) tumors ($P = 0.04$). With the other parameters, similar results were obtained. There was, for instance, a statistically significant association between a high morphological apoptotic index and a low bcl-2 expression ($P = 0.005$) or between a high morphological apoptotic index and a low estrogen receptor status ($P = 0.016$). There was also a statistically significant difference in the survival of patients with tumors showing an increase in apoptosis in the recurrent tumor compared with other tumors ($P = 0.046$, log-rank).

DISCUSSION

In this study, we investigated apoptosis and cell proliferation in breast carcinomas by analyzing specimens from the

Table 1 bcl-2, bax, and estrogen and progesterone receptor expression in prmiary and recurrent tumors

	ER[a]		PR[a]	
	Negative	Positive	Negative	Positive
Primary tumor				
bcl-2				
Weak expression	21	6	23	5
Strong expression	10	13	11	11
P[b]	0.014		0.017	
bax				
Weak expression	21	13	25	9
Strong expression	8	7	10	5
P	0.808		0.799	
Recurrent tumor				
bcl–2				
Weak expression	18	3	20	2
Strong expression	17	12	17	11
P	0.038		0.016	
bax				
Weak expression	19	6	18	7
Strong expression	14	10	20	4
P	0.155		0.273	

[a] ER, estrogen receptor; PR, progesterone receptor.
[b] According to Fisher's exact test.

primary and recurrent breast lesions. The results show that there is a significant increase in apoptosis and proliferation in recurrent tumors compared to the primary tumors. According to the results, the increase in both apoptosis and cell proliferation also influences patient prognosis. In cases in which the increase in apoptosis or proliferation in the recurrent tumor was high, the patients had a shorter survival. This probably depends on alterations in the expression of genes influencing apoptosis or proliferation during tumor progression, which, in turn, also influences the prognosis of the patients.

To study such alterations, we also analyzed changes in the expression of apoptosis influencing bcl-2 and bax proteins and estrogen and progesterone receptors during tumor progression. Genes of the bcl-2 group are known to affect apoptosis, and bcl-2 and bax expression, especially, have been shown to be important in breast carcinoma (18–21, 25). In line with this in all tumor samples, we found a significant inverse association between bcl-2 expression and apoptosis. bcl-2 was also associated with a positive estrogen and progesterone receptor status, both of which also were inversely associated with the extent of apoptosis. The study, thus, supports the previously reported hypothesis that a positive estrogen or progesterone receptor status might up-regulate bcl-2 expression, which could lead to a decrease in apoptosis (26). With bax, no such associations could be found, suggesting that, in breast tumors, bcl-2 expression is more crucial in determining the extent of apoptosis.

When bcl-2 and bax expression in primary and recurrent tumors were compared, a change in their expression was seen in about a half of the cases, and a loss of a positive estrogen or progesterone receptor status was seen in 15%. Cases with an increased bcl-2 expression in the recurrent tumor had a lower level of apoptosis, suggesting that up-regulation of bcl-2 expression in recurrent tumors might lead to a lower level of apoptosis in them. On the other hand, tumors with a loss of a positive estrogen or progesterone receptor status showed an increase in apoptosis and proliferation in the recurrent tumors. The data, thus, show that changes in bcl-2 or the estrogen and progesterone receptor status are parallel to the changes seen in apoptosis or proliferation during breast carcinoma progression.

Here, we also tested whether the location of the recurrence might influence apoptosis or proliferation. Theoretically, this might be possible through interaction of adhesion molecules on the tumor cells with the local matrix. In keeping with this, it has been shown that loss of cellular adhesion by blocking integrin binding to the extracellular matrix by specific antibodies may lead to apoptosis of the cells (27, 28). To study site-specific differences in apoptosis and proliferation, we compared the two phenomena separately in recurrences of the breast scar tissue, lymph nodes, and other sites. Although the extent of apoptosis was higher in all locations compared to the primary tumor lesion, no significant difference was observed in apoptosis between the different recurrent tumor sites, suggesting that the local milieu does not play any significant role in determining the extent of apoptosis in them. On the other hand, the MIB index was significantly higher in recurrent tumors in the breast scar tissue than in other sites, suggesting that cell populations with a higher proliferative activity have a selective advantage over cells with a lower proliferative capacity in this site. Rather than being due to adhesion molecules, this phenomenon may reflect a selective difference between cell populations in local recurrences compared to recurrences in metastatic sites; in cell populations leading to local recurrences, genetic changes favoring an increased cell proliferation could play a more significant role compared to cell populations with a metastatic phenotype.

In our study, apoptosis in the primary breast carcinoma lesions was also associated with a shorter survival of the patients. This is in line with a previous study of Lipponen *et al.* (17), who used only morphology in the assessment of apoptosis. Increased apoptosis is also associated with a shortened survival in other epithelial tumors, such as non-small cell lung carcinomas (15).

In previous reports it was suggested that the *in situ* 3′ end labeling would be unreliable in detecting apoptosis because factors like fixation, DNA damage, or necrosis might influence the results (29, 30). Because of this, we also assessed apoptosis morphologically in H&E-stained slides from the same tumor sections. The results show a strong correlation between morphological apoptosis and the apoptotic index obtained with the 3′ end labeling method. This is in line with the results obtained by other authors (31, 32). Moreover, the morphological apoptotic index showed similar associations as with the 3′ end labeling method. Because the apoptotic index with 3′ end labeling method was slightly higher, it might even be more sensitive in detecting apoptotic cells.

In conclusion, the results show that apoptosis and proliferation are increased during breast carcinoma progression. An increased apoptosis in primary breast carcinoma is associated with a shorter survival of the patients. Furthermore, a significant increase in apoptosis or proliferation in recurrent tumor compared to the primary lesion also predicts a poorer prognosis.

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