Expression of keratinocyte growth factor and its receptor in human breast cancer: its inhibitory role in the induction of apoptosis possibly through the overexpression of Bcl-2

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Summary. Keratinocyte growth factor (KGF), a mesenchymal cell derived paracrine growth factor that regulates normal epithelial cell proliferation, appears to be an essential mediator of steroids in various reproductive organs. The present study was designed to determine the expression and role of KGF and its receptor (KGFR) in human breast carcinoma tissues by immunohistochemistry. We also compared the results with the expression of estrogen receptor α (ERα), ERβ, the proliferative activity assessed by the labeling index (LI) for the Ki-67 antigen, apoptotic frequency assessed by terminal dUTP nick end-labeling (TUNEL) index, and the expression of Bcl-2. All of KGF-positive cases were ERα-positive (p<0.05), but not that of ERβ, while all of KGFR-positive cases were ERβ-positive (p<0.05), but not that of ERα. The specimens with the coexpression of KGF and KGFR significantly correlated with a lower TUNEL index (p<0.05), but not with Ki-67 LI in breast cancer tissues. Further analysis at the cellular level revealed that Bcl-2 was colocalized in KGFR-positive cells, and these cells were almost negative for TUNEL staining. Bcl-2-positive cells were also associated with ERβ, as expected. Therefore, the results indicate that ERα may be involved in KGF expression, and that the coexpression of KGF and KGFR may play an inhibitory role in the induction of apoptosis possibly through the up-regulation of Bcl-2 expression in human breast cancer.

Introduction

The frequency of breast cancer has shown a rapid increase in Japan over the last three decades, with approximately 35,000 new cases per year. Currently, various growth factors and their receptors are known to interact with estrogen in the pathogenesis of breast cancer. Estrogen is a well-known endocrine steroid regulator that modulates the proliferation and differentiation of breast cancer cells, which exerts its action through binding to a specific nuclear receptor, the estrogen receptor α (ERα) (Osborne et al., 2000). Recently, a second isoform of ER, ERβ, was isolated from human tissues (Mosselman et al., 1996). ERα and ERβ are believed to act as an active transcription factor, which binds to specific consensus sequences in genomic DNA—known as the estrogen responsive element (ERE) and the activating protein-1 (AP-1) site—and regulates the transcription of various genes. Paech et al. (1997) reported that the ligand-induced transactivation properties at the AP-1 site differed between ERα and ERβ; 17β estradiol activates the transcriptional function of ERα, whereas it inhibited that of ERβ. In human breast cancer, the expression of ERα and
ERβ was found in the majority of the tumors (Fuqua et al., 2003). While ERα expression is positively correlated with biological parameters of a good prognostic profile, the correlation with ERβ expression and prognosis is highly controversial (Speirs et al., 1999; Fuqua et al., 2003; Jarvinen et al., 2000).

We have already reported that ERα expression may be associated with keratinocyte growth factor (KGF) expression, and that KGF can play an inhibitory role in the induction of breast cancer cell apoptosis (Tamaru et al., 2004). KGF, a unique member of the fibroblast growth factor (FGF) family and also known as FGF-7, is a mesenchymal cell-derived paracrine mediator of epithelial cell growth (Rubin et al., 1989; Finch et al., 1989). A high affinity receptor for KGF (KGFR) was later cloned by Miki et al. and shown to be an alternatively spliced IIIb variant of the FGF receptor (FGFR)-2/bek gene (Miki et al., 1992). In particular, KGF is known to be an essential mediator of various steroid hormones, e.g., andromedin and progestomedin (Yan et al., 1992; Koji et al., 1994). Moreover, KGF is also known to have diverse effects such as the inhibition of apoptosis (Hines et al., 1996; Crescioli et al., 2002), promotion of cell differentiation (Marchese et al., 1997; Yamayoshi et al., 2004), and induction of cell proliferation (Yamamoto-Fukuda et al., 2003). In order to clarify these issues more effectively, we have generated specific polyclonal antibodies against human KGF and KGFR for the immunohistochemical analysis of paraffin-embedded specimens in previous studies (Yamamoto-Fukuda et al., 2003; Yamayoshi et al., 2004; Tamaru et al., 2004).

In general, apoptosis can be controlled by the ratio of various Bcl-2 family members: Certain members promote cell survival (Bcl-2, Bcl-xl, and Bcl-w) while others promote cell death (Bax, Bad, Bak and Bim) (Reed et al., 1996; Adams et al., 1998; Koji and Hishikawa, 2003). Among this family, an elevated level of Bcl-2 leads to an extended survival of cells and, inversely, an increased level of Bax accelerates cell death in various organs (Baba et al., 1999; Sun et al., 2002). In breast cancer, Bcl-2 has also been demonstrated to inhibit apoptosis, and the ratio between Bcl-2 and Bax seems to be an important determinant of induction of apoptosis (van Slooten et al., 1996; van Slooten et al., 1998; Charpin et al., 1998). However, the exact biological significance between KGF and/or KGFR expression and Bcl-2 expression remains largely unknown in human breast cancer.

The present study therefore compared the expression of KGF and KGFR with the ERα and ERβ expression, proliferative activity, and frequency of apoptosis, and then addressed whether the KGF and KGFR expressions were correlated with Bcl-2 expression, mimicking the frequency of the cancer cell apoptosis. Consequently, the results indicated an involvement of the KGF system in the inhibition of the induction of apoptosis, possibly in association with Bcl-2, in human breast cancer.

Materials and Methods

Biochemicals and chemicals

Paraformaldehyde (PFA) was purchased from Merck (Darmstadt, Germany). 3,3’-diaminobenzidine/4HCl (DAB) was purchased from Doyin Laboratories (Kumamoto). Biotin-16-dUTP and terminal deoxynucleotidyl transferase (TdT) were purchased from Roche (Mannheim, Germany). 3-aminopropyltriethoxysilane, Brij 35, bovine serum albumin (BSA, minimum 98%, electrophoresis) were purchased from Sigma Chemical (St. Louis, MO, USA). All other reagents used in this study were purchased from Wako Pure Chemicals (Osaka) and were of analytical grade.

Antibodies

Polyclonal antibodies against KGF and KGFR were prepared by immunizing rabbits against synthetic peptides in cooperation with Nichirei Co. (Tokyo) as previously described (Yamamoto-Fukuda et al., 2003; Tamaru et al., 2004; Yamayoshi et al., 2004). For immunohistochemical analysis, anti-KGF IgG (5 µg/ml) and anti-KGFR antiserum (1:600) were used because of the highest signal/noise ratio in the immunostaining of paraffin-embedded sections of human breast cancer tissues.

A mouse monoclonal antibody against human ERα (0.3 µg/ml) was purchased from Dako (Glostrup, Denmark). A rabbit polyclonal antibody against human ERβ (1 µg/ml) was purchased from BioGenex (San Ramon, CA, USA). A mouse monoclonal antibody against human Ki-67 (0.5 µg/ml) was purchased from Immunotech (Marseille, France). A rabbit polyclonal antibody against human Bcl-2 (2 µg/ml) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG F(ab)’ (1:100) was purchased from Chemicon International (Temecula, CA, USA). HRP-conjugated goat anti-rabbit IgG F(ab)’ (1:200) was purchased from Medical and Biological Laboratories (Nagoya). HRP-conjugated goat anti-biotin was purchased from Vector Laboratories (Burlingame, CA, USA).

Breast cancer patients and tissue preparation

Tumor specimens were obtained from 21 patients with human breast cancer undergoing surgical treatment...
from 1998 to 2001 in the Second Department of Shimane Medical University. Totally, 18 cases were an invasive ductal carcinoma, and 3 cases were an invasive lobular carcinoma. All patients were female, and the average age was 59.1 ± 14.6 years (mean ± SD). No patients were treated with any preoperative chemotherapy. As for menopausal status, 8 patients were premenopausal, and 13 patients were postmenopausal. All patients or their next of kin provided informed consent for participation in the clinical study. The tissues were fixed with 4% PFA in phosphate-buffered saline (PBS) and embedded in paraffin. Serial sections were cut at a 5 μm thickness and then placed onto 3-aminopropytriethoxysilane-coated glass slides.

**Immunohistochemistry**

Enzyme immunohistochemistry was performed to examine the expression of KGF, KGFR, ERα, ERβ, Bcl-2, and Ki-67 in breast cancer tissues as previously described (Damarvandi et al., 2002; Yamamoto-Fukuda et al., 2003; Hishikawa et al., 2003). For KGFR detection, the sections were pretreated with 0.2% Triton X-100 in PBS for 10 min at room temperature (RT). For the immunohistochemistry of ERα, ERβ, and Ki-67, the sections were autoclaved at 121°C for 15 min in a 10 mM citrate buffer (pH 6.0). After inactivation of endogenous peroxidase activity with 0.3% H2O2 in methanol for 15 min at RT, the sections were preincubated with a blocking solution for 1 h at RT. For KGFR, 10% normal goat serum and 1% BSA in PBS was used, and for the others, 500 μg/ml normal goat IgG and

![Fig. 1. Immunohistochemical detection of KGF (a), KGFR (b), ERα (c), and ERβ (d) in paraffin sections of invasive ductal breast cancer tissues. Bar=100 μm](image-url)
1% BSA in PBS was used as a blocking solution. The sections were then incubated with the first antibodies for 2 h (KGF, KGFR, and Bcl-2) or overnight (ERα, ERβ, and Ki-67) at RT. After the slides were washed with 0.075% Brij 35 in PBS, the HRP sites were visualized with DAB and H2O2 solution or DAB, Ni, Co, and H2O2 solution according to the method by Adams (1981). For a negative control, either normal rabbit IgG, normal rabbit serum or normal mouse IgG was used instead of the first antibody in every experiment.

Terminal dUTP nick end-labeling (TUNEL) staining
To identify nuclei with DNA strand breaks at the cellular level, TUNEL was performed according to the method by Gavrieli et al (1992) with a slight modification (Yamamoto-Fukuda et al., 2000; Koji et al., 2001). Briefly, the sections were deparaffinized with toluene and rehydrated in serially graded ethanol solutions. After washing with PBS, the sections were treated with 0.1 μg/ml of proteinase K in PBS at 37°C for 15 min. The sections were then rinsed once with distilled water and incubated with TdT buffer (25 mM Tris-HCl buffer, pH 6.6, containing 0.2 M potassium cacodylate and 0.25 mg/ml BSA) alone for 30 min at RT. After incubation, the sections were reacted with 200 U/ml TdT dissolved in a TdT buffer supplemented with 5 μM biotin-16-dUTP, 20 μM dATP, 1.5 mM CoCl2, and 0.1 mM dithiothreitol at 37°C for 90 min. Then the reaction was terminated by washing with a 50 mM Tris/HCl buffer (pH 7.5) for 15 min. Endogenous peroxidase activity was inhibited by immersing the slides in 0.3% H2O2 in methanol for 15 min at RT. The signals were detected immunohistochemically with an HRP-conjugated goat anti-biotin antibody.

Quantitative analysis
The results of immunohistochemistry for KGF, KGFR, ERα, ERβ, Bcl-2 and Ki-67 were graded as positive or negative, compared with the staining of the control group. For the quantitative analysis of Ki-67, more than 2000 cells were counted in random fields at X400 magnification, and the number of Ki-67-positive cells was expressed as a percentage of positive cells per total number of counted cells (Ki-67 LI; mean ± SD). The percentage of TUNEL-positive cells was calculated in the same manner as in the case of Ki-67 LI.

Statistical analysis
Mean values were compared with an unpaired Student’s t-test, and categorical variables were compared with χ² analysis. A p value<0.05 was considered statistically significant. All analyses were performed with a statistical software package (StatView, version J 5.0; Abacus Concepts, Berkeley, CA, USA).

Fig. 2. Immunohistochemical detection of Bcl-2 in paraffin sections of invasive ductal breast cancer tissues. a: Bcl-2 immunostaining in the non-cancerous lesion. b: Bcl-2 immunostaining in the cancerous lesion. Bar=100 μm
Results

Expressions of KGF, KGFR, ERα, and ERβ, and their correlation with cell kinetic parameters in human breast cancer

KGF was localized in the cytoplasm of cancer cells and some stromal cells in 9 out of 21 patients (Fig. 1a). KGFR was localized in the plasma membrane and cytoplasm of cancer cells in 10 out of 21 patients (Fig. 1b). In total, 6 cases were KGF- and KGFR-negative, 6 cases were KGF-negative but KGFR-positive, 5 cases were KGF-positive but KGFR-negative, and 4 cases were both KGF- and KGFR-positive. ERα was detected in the nuclei of cancer cells in 16 out of 21 cases (Fig. 1c), while ERβ was detected in the nuclei of cancer cells in 17 out of 21 cases (Fig. 1d). All of KGF-positive cases were ERα-positive (p<0.05), but there was no correlation between KGF expression and ERβ expression. All of KGFR-positive cases were ERβ-positive (p<0.05), but there was no correlation between KGFR expression and ERα expression. Next, TUNEL staining and Ki-67 immunostaining were performed to evaluate the correlation with KGF and/or KGFR expression. The percentage of TUNEL-positive...
cells in the KGF and KGFR double-positive cases was significantly lower than that of any other combinations (1.1 ± 0.6% versus 3.1 ± 1.7; p<0.05). No correlation was found between the expression of KGF and/or KGFR and Ki-67 LI.

**Correlation between KGF and/or KGFR expression and Bcl-2 expression**

To gain insights into the mechanism underlying the correlation between KGF and/or KGFR expression and the inhibition of apoptosis, we performed an immunohistochemical analysis of the Bcl-2 expression. In the non-cancerous lesion of human breast cancer, Bcl-2 was only weakly expressed in the ductal cells and some stromal cells, while Bcl-2 was over-expressed diffusely in the cytoplasm of cancer cells in 11 out of 21 patients (Fig. 2a). As a negative control, the section was reacted with normal rabbit IgG, and no staining was found (data not shown). Eight out of 11 Bcl-2-positive cases were KGFR-positive, and 8 out of 10 Bcl-2-negative cases were KGFR-negative (p<0.05). No significant correlation was found between KGF and Bcl-2 expressions.

To clarify the direct relationship between KGFR-positive cells and Bcl-2-positive cells, we performed immunostaining for KGFR and Bcl-2 in mirror sections of human breast cancer tissues. As clearly shown in Figure 3, the cells positive to KGFR were almost positive to Bcl-2 (Fig. 3a, b), while the cells negative to KGFR were also negative to Bcl-2 (Fig. 3d, e). In addition, no TUNEL-positive cells were found in the areas consisting of Bcl-2-positive cells (Fig. 3c). In contrast, TUNEL-positive cancer cells were frequently found in the negative areas (Fig. 3f).
Correlation between Bcl-2 and ERs expressions

To evaluate the association between the expressions of Bcl-2 and ERs, we performed immunostaining for Bcl-2 and ERs in mirror sections of human breast cancer tissues. All of Bcl-2-positive cases were ERβ-positive, and 4 of 10 Bcl-2-negative cases were also ERβ-negative (p<0.05). As shown in Figure 4, almost all Bcl-2-positive cells expressed ERβ (Fig. 4a, b), but Bcl-2 negative cells were also negative to ERβ (Fig. 4c, d). In addition, there was no correlation between the expressions of Bcl-2 and ERα (data not shown).

Discussion

In the present study, we first investigated the expression of KGF and KGFR in human breast cancer tissues, and then its correlation with ERα and ERβ expressions. The results indicated that KGF and KGFR expressions were significantly correlated with ERα and ERβ expressions, respectively, and that the coexpression of KGF and KGFR was associated with a low frequency of apoptotic cancer cells. To gain insights into the inhibitory mechanism of the cancer cell apoptosis, we examined the involvement of Bcl-2 in the same set of specimens, and found a very close association of Bcl-2 overexpression with KGFR expression at both cellular and tissue levels.

It has been suggested that the rate of growth of breast cancer is determined by the balance of cell proliferation and cell death. In the present study, we confirmed that the coexpression of KGF and KGFR was significantly correlated with a lower frequency of apoptosis, but not with Ki-67 LI in the breast cancer. This finding is not surprising since it has been known that KGF plays various roles in the regulation of cell kinetics. An antiapoptotic effect of KGF was also reported in human prostate cancer cells, as well as the intestinal epithelium of the total parenteral nutrition model mouse (Crescioli et al., 2002; Wildhaber et al., 2003). Previously, we also reported that KGF inhibited the induction of apoptosis by anticancer drugs such as 5-fluorouracil or cyclophosphamide in MCF7 cells in vitro (Tamaru et al., 2004). Moreover, in salivary adenocarcinoma, KGF may inhibit tumor growth though the induction of apoptosis (Zhang et al., 2001). On the other hand, Yamayoshi et al. (2004) reported that the coexpression of KGF and KGFR correlated with a high Ki-67 LI in lung adenocarcinoma with a poor prognosis, but not in lung squamous cell carcinoma, indicating different roles according to the cell-type. Therefore, although we do not have a precise explanation for the exact mechanism of KGF actions at present, the KGF system may be involved in the regulation of tissue mass across a wide variety of biological and pathological aspects.

Bcl-2 appears to be of particular importance since it is known to be a key factor in maintaining cell survival in various tumors including prostate and lung cancers (McDonnell et al., 1992; Ikegaki et al., 1994). However, the role of Bcl-2 in human breast cancer seems to be more complicated (Johnston et al., 1992; Leek et al., 1994; Sierra et al., 1995; Hellemans et al., 1995). While breast cancers expressing high levels of Bcl-2 were reported to show low rates of apoptotic cell death, they also have low proliferation rates, low histopathological grades, and improved prognoses (Charpin et al., 1998; van Slooten et al., 1998). Strong Bcl-2 expression was also associated with ER- and progesterone receptor-positive statuses, both of which also were reversely correlated with the apoptotic index and were also related to a favorable prognosis (Bhargava et al., 1994; Lipponen P et al., 1995). Recently, ERβ has been localized in mitochondria, suggesting that estrogen can directly affect mitochondrial function through ERβ (Yang et al., 2004; Chen et al., 2004). Bcl-2 is thought to be localized in the inner mitochondrial membrane, the nuclear envelope, and the endoplasmic reticulum, and it has been shown to influence the mitochondrial membrane potential and regulate the cellular process of apoptosis (Hockenbery et al., 1990). Interestingly, in the present study, Bcl-2 expression was closely associated with KGFR-positive cells, and was also correlated with ERβ-positive cells. Therefore, KGFR expression may be involved in the regulation of apoptosis related to the elevation of Bcl-2 expression under the influence of estrogen through ERβ in human breast cancer, although further analyses would be needed to clarify each role or interaction of these molecules.

In our previous and present studies, ERα expression was significantly associated with KGF expression (Tamaru et al., 2004). We have also reported that, although the promoter of the KGF gene has ERE sequences, the expression of KGF in ERα-positive human breast cancer cell lines was not affected significantly by the presence or absence of estrogen, and so the regulation of the KGF expression by ERα may be in a ligand-independent manner (Tamaru et al., 2004). For instance, the members of the epidermal growth factor and insulin-like growth factor (IGF) can activate ERα by directly phosphorylating crucial residues (Kato et al., 1995). There is also considerable cross-talk between ERα and IGF signal transduction pathways. Further studies may provide clues to the possible mechanism of ERα in a ligand-independent manner in human breast cancer.

In conclusion, we have found that the coexpression of KGF and KGFR, which seemed to be correlated with ERα and β expressions, respectively, may play an inhibitory role
in the apoptosis of human breast cancer cells through the up-regulation of Bcl-2 expression. Considering the mitochondrial localization of ERβ, analysis of the KGF signaling cascade in association with mitochondrial apoptosis-related components would be helpful to understand the complicated functions of the KGF system.

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