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Enhanced Expression of Neuropeptides in Human Breast Cancer Cell Lines Following Irradiation

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AALTO, Y., S. FORSGREN, U. KJÖRELL, J. BERGH, L. FRANZÉN AND R. HENRIKSSON. *Enhanced expression of neuropeptides in human breast cancer cell lines following irradiation.* PEPTIDES. **19**(2) 231–239, 1998.—Previously, we have observed that the expression of the neuropeptides bombesin (BN-), the mammalian counterpart being gastrin-releasing peptide (GRP), and substance P (SP) in intact normal tissues, such as salivary and laryngeal glands, increases in response to irradiation. In the present study, the aim was to evaluate whether irradiation can have effects on individual cells that normally synthesize neuropeptides. In addition, since these neuropeptides are potentially mitogenic, we studied tumor cells. Therefore, the estrogen receptor-negative human breast cancer cell line MDA-MB-231 and its subline, with acquired doxorubicin resistance, MDA-MB-231 Dox were examined before irradiation and 4, 10, and 15 days after irradiation with 4 Gy (195 kV, 2 Gy fractions with 4 hours interval). Potential dose related changes were studied by delivering single doses of 2 or 9 Gy with the same technique. Immunohistochemical and radioimmunoassay (RIA) methods were used for detection of the SP and BN/GRP. Before, and at all time points following irradiation, a subpopulation in both cell lines displayed an intense immunostaining of SP and BN/GRP. A partial reorganization of the immunoreactive material was observed 10 days after irradiation. The RIA-analyses displayed signs of a dose-related increase, and a time-dependent transient and significant increase in the content of both peptides. The pattern of changes differed between the two peptides, and was especially pronounced in the doxorubicin resistant cells with regard to SP. Another neuropeptide, calcitonin gene related peptide (CGRP), was not detected in the cells used. The results suggest that irradiation has effects on a population of cultured neuropeptide-synthesizing cells. The occurrence and the specific changes obtained in the levels of neuropeptides, in response to irradiation, might imply an importance in the growth of breast cancer cells and in explaining repair processes following irradiation. © 1998 Elsevier Science Inc.

Irradiation Growth factor Bombesin Substance P Breast cancer

WE have previously demonstrated specific dose- and time-dependent alterations in the expression of the neuropeptides bombesin (BN) and substance P (SP) in normal rat tissues such as salivary glands (1,11,14), and submucosal glands in larynx (22,23) following irradiation of intact animals. It is not known whether irradiation influences neuropeptide expression in individual neuropeptide-containing tumor cells and/or in intact tumours to the same extent. That includes breast carcinoma, in which tumor cells express various neuropeptides (34). Some of the neuropeptides that occur in breast carcinoma, like the amphibian tetradecapetide BN [the mammalian counterpart being gastrin-releasing peptide (GRP)], and SP have been reported to be mitogenic for various cell-types including tumor cells (5,7,37,42). BN/GRP may be involved in the function and growth of human breast cancer cells (8,16,36,40,45). Accordingly, BN/GRP receptors have been detected in human breast carcinoma cells (20).

The purpose of the present study was two-fold: To examine if, and how, irradiation can change the expression of SP and BN/GRP in individual cells, and to evaluate whether the level of these neuropeptides, acting as growth factors, could be modified by irradiation of tumor cells. Therefore, we found it of interest to use the human breast cancer cell

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line MDA-MB-231 and the corresponding multidrug resistant subline (MDA-MB-231 Dox), adapted to growth in continuous presence of the chemotherapeutic doxorubicin.

METHOD

Cell Lines and Tissues

Human breast adenocarcinoma cell lines MDA-MB-231 were cultured in RPMI 1640 and 10% fetal calf serum, in a humidified atmosphere of 5% CO_2/95% air at 37°C with medium change twice a week. The MDA-MB-231 cell line became resistant to doxorubicin by adding increasing concentrations of doxorubicin (Dox) to the medium in the selection procedure. The status, including viability of the cells, was monitored twice a week. The doxorubicin concentration was gradually increased to 75 ng/ml. The MDA-MB-231 Dox cells demonstrated features of classical multidrug resistance (MDR-1) with an increased expression at the mRNA level and an enhanced expression of glycoprotein p-170 (not shown).

Specimens of rat larynx and tibialis anterior muscle were also processed for immunohistochemistry, in order to provide sections to be used in control staining. For this purpose, adult female rats were anaesthetized with sodium pentobarbital (40 μg/kg i.p.), and the larynx and the tibilis anterior muscle were dissected out.

Experimental Preparation

Both the MDA-MB-231 cell line and the doxorubicin resistant MDA-MB-231 Dox cell line were irradiated. Irradiation was delivered with 195 kV X-ray at 22°C using a 0.5 mm Cu filter (Stabilipan, Siemens, Germany). The source-phantom distance was 500 mm, and the dose rate was 1 Gy/min at the level of the irradiated cells. The radiation dose was 2 Gy in two factions, delivered with an interval of four hours. The petri dishes were put on a 150 mm thick lucite block, in order to allow full backscatter. After irradiation, the cells were incubated as described above. Non-irradiated cells were handled in parallel. Four, 10 or 15 days after irradiation, cells and corresponding controls were harvested. For each group, radioimmunoassay and immunohistochemistry were carried out, as outlined below. The same technique was also used to evaluate any dose-response relations by analyzing cells ten days after delivering single doses of 2 or 9 Gy.

Immunohistochemical Procedures

The cells from each group [non-irradiated cells at each time point (''controls''), and 4, 10, or 15 days after irradiation] were harvested after trypsinizing and suspended in a balanced salt solution to a concentration of 10^6 cells per ml, after counting by using a cell counter (Coulter Counter Multisizer®). The slides were pre-coated with chrome-alum gelatin, and cells were collected by a cytocentrifuge (Cytospin Shandon, Southern Ltd, Runcorn England) at 96 g for 5 min giving approximately 50 000 cells/slide. The sections were then fixed by immersion overnight at 4°C in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, whereafter the sections were thoroughly washed in Tyrode's solution, containing 10% sucrose, at 4°C overnight. The sections were kept at −18°C and then processed for immunofluorescence. The immunohistochemical procedures were as described previously (10). The sections were incubated for 30 min in a 1% solution of detergent Triton X-100 (Kebo Lab, Stockholm, Sweden), in 0.01 M phosphate buffered saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative, rinsed three times for 5 min each in PBS, and incubated in 5% normal swine serum in PBS supplemented with 0.1% bovine serum albumin (BSA) for 15 min. The sections were thereafter incubated with the primary antibody, diluted in PBS with BSA, in a humid environment. Incubation was performed for 60 min at 37°C. After incubation with specific antiserum and after three 10 min washes in PBS, the sections were immersed in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark), diluted 1:40, at 37°C in a moist chamber, washed in three changes of PBS, mounted in glycerol:PBS (1:1), and examined under a Leitz Orthoplane Photomicroscope equipped with epifluorescence optics.

In order to relate the immunoreactivity to cellular morphology, some sections were first immunostained as described above and photographed. Thereafter, the sections were eluted with acid potassium permanganate for 2 min and stained with May Grünewald Giemsa. The rat larynx specimens examined in parallel were fixed and rinsed, as were the cells described above. The specimens were mounted in embedding medium and frozen in propane chilled with liquid nitrogen, whereafter they were sectioned in a cryostat at −25°C.

Antibodies

The BN/GRP antibody had been raised in the rabbit using synthetic amphibian bombesin conjugated with bovine serum albumin, glutaraldehyde being the coupling agent (Amersham, Buckinghamshire, UK; working dilution 1:40). The antibody shows 100% cross-reactivity with BN 1-14, and the cross-reactivity with synthetic GRP is 95%, and that with porcine GRP 92%. It shows 91% cross-reactivity with BN 4-14 and 83% cross-reactivity with BN 5-14, while cross-reactivity with BN 9-14 is 28% and that with BN 7-11 and BN 2-5 is <0.01%. The antiserum shows 0.2% cross-reactivity with substance P. The BN/GRP antibody is frequently used in our laboratory, and its specificity is continuously characterized via incubations of sections of formaldehyde-fixed rat tissue; preincubation of the antibody with synthetic BN completely abolishes specific immunoreaction, but also preabsorption with substance P yields partial disappearance of immunoreaction (23). Therefore,

batches of BN-antiserum preabsorbed with substance P were used for the staining of the two MDA-MB-231 cell lines.

Rabbit antibodies against substance P (working dilution: 1:200, code i675/002, UCB, Brussels, Belgium), and calcitonin gene related peptide (CGRP) (working dilution: 1:100, code RPN 1842, Amersham Int, Buckinghamshire, UK) were also used. Preabsorption tests of the batches of SP-antibody used for staining of the cells were done to confirm the characteristics of the antibody by the following control procedure: Sections of specimens of the rat larynx were stained with the SP-antibody or with SP-antibody preabsorbed with SP (Sigma) (20 μg/ml antiserum). Staining with SP-antibody gave similar results as obtained in previous SP-studies on rat larynx in our laboratory, whereas the immunoreaction was completely abolished in sections of larynx stained with SP-antibody preabsorbed with SP. The CGRP-antibody was also tested by preabsorption tests of tissue (rat larynx) sections. The SP-(UCB), BN/GRP-(Amersham), and CGRP-(Amersham) antibodies are all directed against fully processed peptides.

Staining with only normal serum did not reveal specific staining. As an extra control, MDA-MB-231 cells were stained with an antibody directed against a non-peptide antigen (myosin), the effectiveness of this staining being tested by parallel processing of rat skeletal muscle (tibialis anterior). No specific staining was observed in the MDA-MB-231 cells by use of this antibody.

Radioimmunoassay (RIA)

The frozen cell pellets were lyophilized, and the samples were then reconstituted and diluted in BSA buffers and homogenized by vortexing. After centrifugation, the supernatants were assayed for the peptides in at least two different dilutions. Non-irradiated control cells were continuosly analyzed from each separate time point. The concentrations of BN- and SP-like peptide in the extracts were determined by using RIA kits (^{125}I) purchased from Incstar Corp (Stillwater, Minnesota, USA). The RIAs were performed according to the instructions from the manufacturer. The BN-antibody was directed against BN isolated from the frog skin and has been tested by Incstar to display 50% crossreactivity with porcine GRP but only $<0.002\%$ crossreactivity with other peptides tested (including substance P). The SP-antibody has been found to show 100% cross reactivity with SP but 0.008% or less reactivity with other peptides. Cells from 3–8 separate experiments were analyzed.

RESULTS

Immunohistochemistry

BN/GRP. Some cells in both the sensitive and the resistant cell lines displayed BN/GRP-like immunoreactivity (LI). BN/GRP-LI was detected both after irradiation as well as in the non-irradiated controls. The BN/GRP-LI occurred in the cytoplasm, mostly appearing as a diffuse staining involving a large part of the cytoplasm (Figs 1 and 2). In other occasions, the BN/GRP-LI occurred as small granular spots that were scattered in the cytoplasm. These latter appearances were particularly observed 10 days after irradiation (Figs 3 and 4). The staining was verified to be cytoplasmatic by examination after sequential double-staining of the cells; the cells first being stained for BN/GRP, and then with May Grünewald Giemsa (Fig 5).

SP. Cells in both the controls and 4, 10 and 15 days after irradiation also showed intense SP-immunoreactivity. This was the case in both the MDA-MB-231 cell line and the MDA-MB-231 dox cells. The immunoreaction mainly appeared as coalescing immunoreactive material in parts of the cytoplasm (Fig 6). The clumps of immunoreactive material were of very varying sizes, from being very small to comprising a major part of the cytoplasm (Figs 6, 7). In a few occasions, the immunoreactive material surrounded the nucleus. Ten days after irradiation of both cell lines the pattern of immunoreaction was partly different: numerous fine immunoreactive spots were observed in the cells of these stages (Fig 8). The aggregates of coalescing immunoreactive material stood out very clearly at low magnification (Figs 9, 11). As was observed for BN-staining, the cytoplasmic location of the SP immunoreaction was verified (Fig 10).

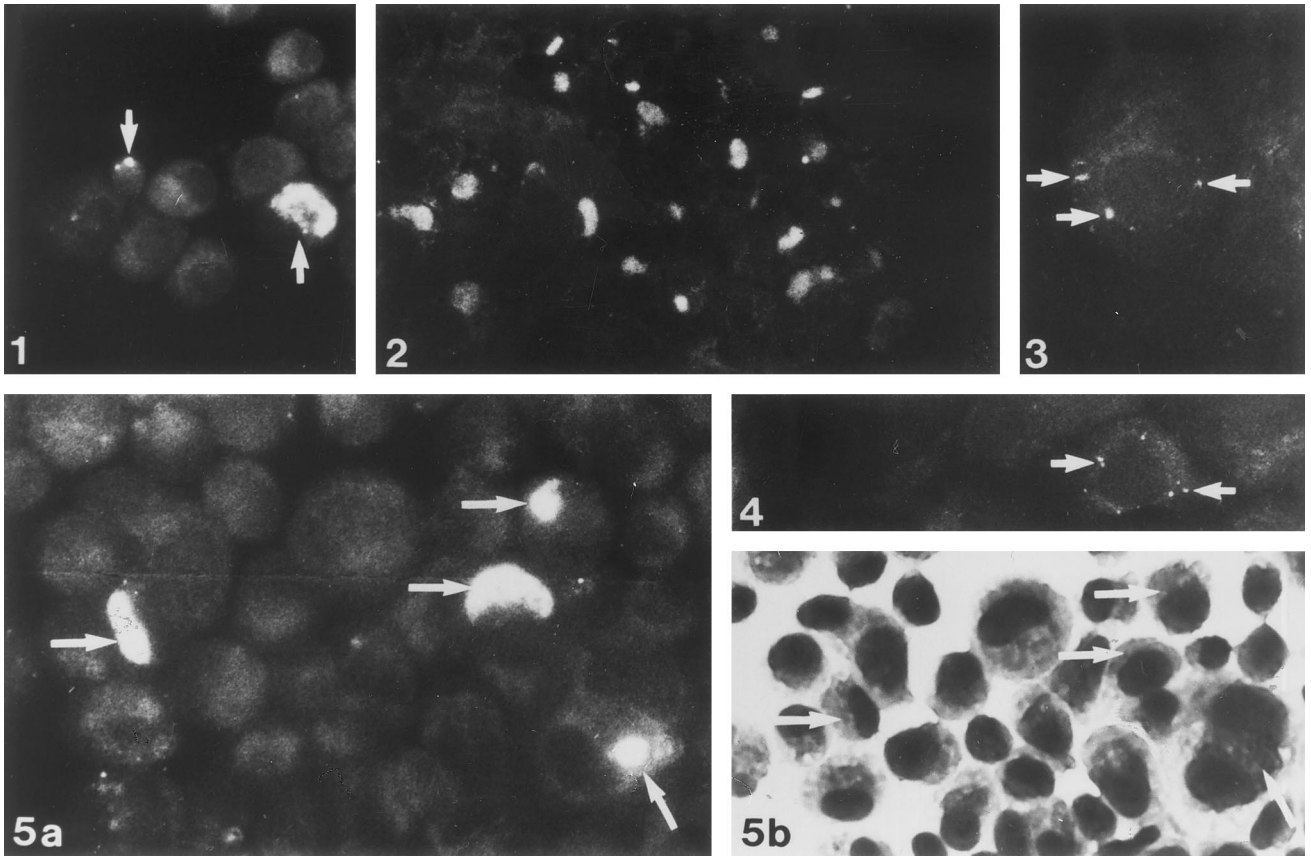
The doxorubicin resistant cells in general displayed similar appearances as the MDA-MB-231 cells at all stages after staining for BN/GRP (Figs 1–5) as well as SP (Figs 6–11). However, the intensity of immunoreaction appeared to be more pronounced in the doxorubicin resistant cells. There were no obvious differences between non-irradiated controls handled in parallel and analyzed at the various time points.

CGRP. No cells at any stage of any of the cell lines studied showed specific immunoreactivity (Fig 12).

RIA

The RIA analysis revealed a time-related increase in BN/GRP concentrations in both the MDA-MB-231 Dox resistant and sensitive cell lines after irradiation (2×2 Gy) (for details, see Fig 13). A dose-related response was evident in especially the doxorubicin sensitive cell line after delivery of single doses of 2 and 9 Gy (Fig 14). There were no statistical differences between non-irradiated controls handled in parallel and analyzed at different time points after irradiation, with regard to respective cell line (not shown).

Time-dependent changes were also seen with respect to SP-like peptides, in both cell cultures, by RIA. In the MDA-MB-231 cell line, mean SP concentrations of 30.8 pg/10^6 cells, 38.8 pg/10^6 cells, 34.5 pg/10^6 cells, and 41.5 pg/10^6 cells were found in the control and 4, 10 and 15 days after 2×2 Gy irradiation, respectively. A similar, however more pronounced, increase was seen in the MDA-MB-231



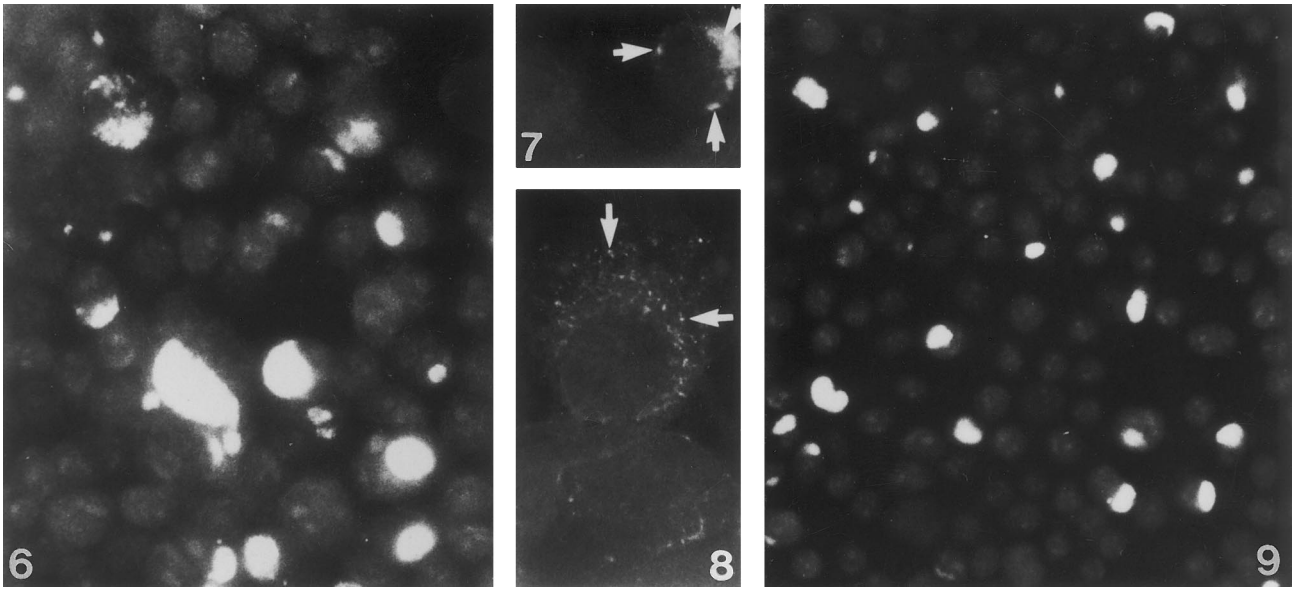
FIGS. 1–5. 1 and 2, MDA-MB-231 Dox cell lines processed for BN/GRP. Examination at 4 days after irradiation. A large partly granular mass and smaller granular structures are immunolabelled in (1) (arrows). In (2) the cells are shown at lower magnification: Several of the cells show immunoreactivity. 1) ×400, 2) ×250. 3 and 4, cells of MDA-MB-231 Dox cell lines 10 days after irradiation. Incubation with BN/GRP-antibody. Small immunoreactive spots are observed (arrows). ×640. 5, MDA-MB-231 cell line in a section first incubated with BN/GRP-antiserum (a) and then stained with May Grünewald Giemsa (b). Some of the cells show immunoreactivity, confined to parts of the cytoplasm. Arrows point at corresponding regions. a) ×900, b) ×750.

doxorubicin resistant cell line, the concentrations being 27.4 pg/10^6 cells, 41.5 pg/10^6 cells, 53.6 pg/10^6 cells, and 55.4 pg/10^6 cells in the control and 4, 10 and 15 days after irradiation, respectively (see also Fig 15). A dose-related response was indicated following single doses of radiation between the radiation dose-interval 2–9 Gy (Fig 16).

DISCUSSION

The present study shows that a subpopulation of the cells, both in the doxorubicin resistant and the sensitive breast carcinoma cell lines (MDA-MB-231), displayed BN/GRP- and SP-LI, which is in accordance with previous observations on breast carcinoma cells (34). The results of the study also suggest that irradiation induced an increase in the expression of both BN/GRP- and SP-like peptides, as verified by the RIA analysis. This increase seen in response to irradiation, at least with regard to SP-like peptide, was found to be most pronounced in the doxorubicin resistant cells. The effects seem to be time-dependent and dose-related. Furthermore, a reorganization of the immunoreactive BN/GRP- and SP-material was found to occur, i.e., 10 days after irradiation the immunoreaction mainly appeared as fine immunoreactive spots, whereas the immunoreaction at the other stages appeared as aggregates of coalescing material.

Our data demonstrate the capacity of irradiation to directly stimulate a human breast cancer cell line to increase the level of the investigated neuropeptides above the basal level of non-irradiated controls, after a relatively modest dose of 2 × 2 Gy. Another important finding is the time-dependency in the change of peptide content in response to irradiation seen. The irradiation-induced effects are likely to be related to an effect on all the cells in the cell line; a change in inter-communication between the various cells and/or changes in the production of chemical substances may be responsible for the increase in neuropeptide synthesis seen. In comparison, also in organs of intact animals in which neuropeptide expression has been found to be



FIGS. 6–9. 6, nonirradiated MDA-MB-231 Dox cell line processed for SP. Several of the cells show intensely fluorescent aggregates of immunoreactive material. These aggregates are of varying dimensions. ×310. 7 and 8, cells of MDA-MB-231 Dox cell line examined 15 days after irradiation (7) and MDA-MB-231 cell line examined 10 days after treatment (8). Staining for SP. The cell in (7) shows coalescing fluorescent material (arrows) in its periphery, the cell in (8) shows immunoreactive fine spots (arrows). 2. ×400, 3. ×640. 9, Non-irradiated MDA-MB-231 Dox cell line, in a section processed for SP. Several of the cells show an intense fluorescence reaction confined to parts of their cytoplasm. ×250.

changed in response to irradiation, marked changes at the cellular levels occur. These changes might lead to an altered production of neurotrophic factors and/or other chemical messenger substances which secondarily can affect the innervation in the organ; we have no proofs of the occurrence of direct effects on the neurons (12,14). Accordingly, in addition to the direct effects on tumor cells, induced growth factors in adjacent normal tissues (17) indirectly may have the capacity to stimulate tumor growth through intercellular communications. It should also be stressed that the increase in the peptide levels shown by RIA conforms to the levels in the whole cell line. In further studies, in which the

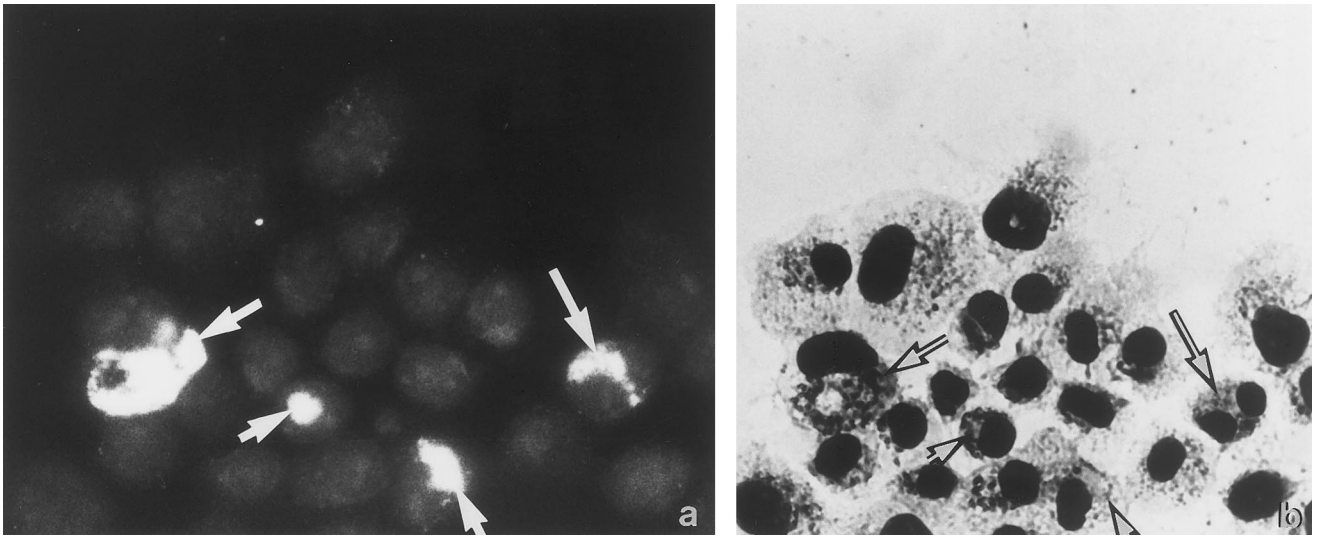
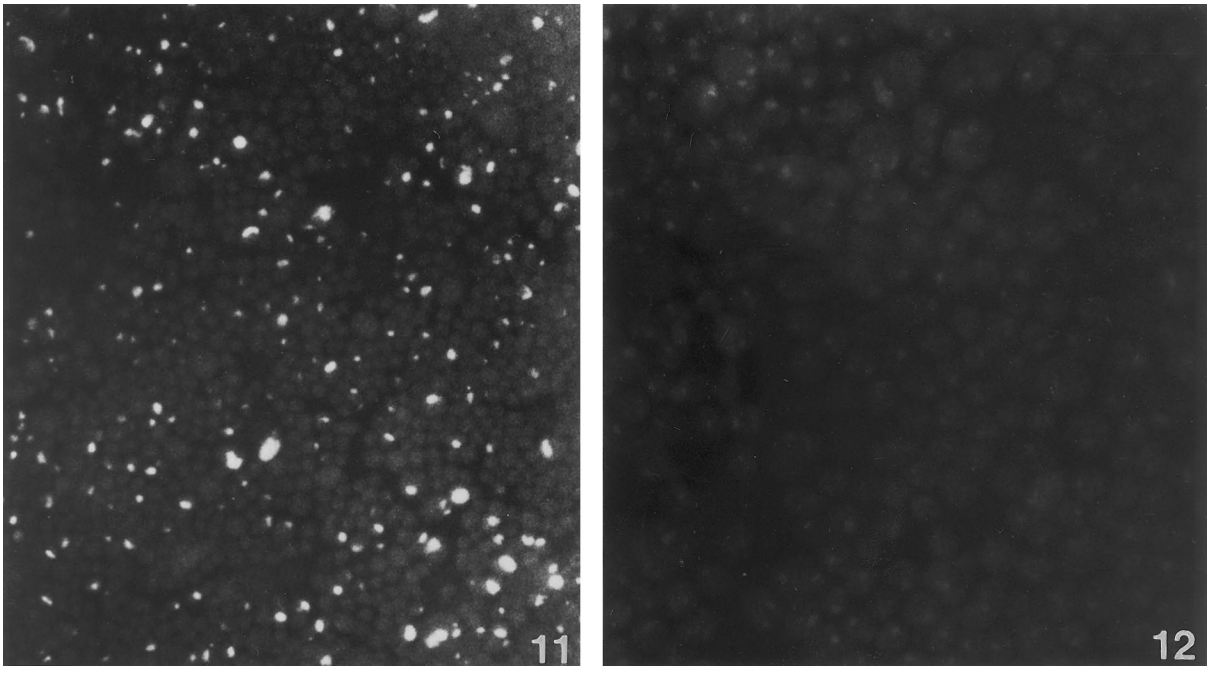


FIG. 10. A non-irradiated MDA-MB-231 cell line first processed for SP (a) and then stained with May Grünewald Giemsa (b). The locations of the SP-immunoreactive aggregates seen in (a) are also marked in (b) (arrows). a) ×900, b) ×750.



FIGS. 11 AND 12. 11, A MDA-MB-231 Dox cell line processed for SP, shown at low magnification. ×125. 12, A MDA-MB-231 cell line processed for CGRP. None of the cells are immunolabelled. ×310.

peptide levels in conditioned media are examined, issues concerning the degree of peptide secretion might be solved.

The changes in BN/GRP- and SP-levels seen in the present study are consistent with previous findings that synthesis of neuropeptides like BN/GRP and SP are increased in organs, after irradiation to a whole region of the body in animals with an intact nervous system, that this increase occurs in cells (neurons) that already have the capacity to synthesize the peptide, and that the changes in expression of the peptides are time dependent (1,11,14,22). This could imply that a single stimulus such as irradiation, by inducing an effect in a target organ, sets in motion a coordinated series of changes in synthesis of molecules in the nervous system of this organ and/or in the population of peptide-containing cells in the particular organ/tumor, which then is reversed when the signal pathways of regeneration are completed. Consequently, the increase in peptide content might result from a radiation induced increase in de novo synthesis of the peptide by cells which survived the lethal effects of radiation, remained metabolically active, and which had the potential to synthesize the peptide. In addition to influence the rate of synthesis or the nature of the factors produced, radiation-exposure may also alter the ability of specific cell types to respond to signals from these factors. Such alterations may occur through a change in the number or the affinity of receptors on the target cells. Indeed, BN/GRP-like receptors have been demonstrated in the human breast tumor cell lines, such as the MDA-MB-231 cell line used in the present study (16,29), and recently BN/GRP-receptors were demonstrated in specimens of human breast cancer (20). It was even suggested that BN/GRP antagonists might be useful in the treatment of breast cancer. It is also obvious that the radiation induced increase in the expression of BN/GRP- and SP-like peptides is not a

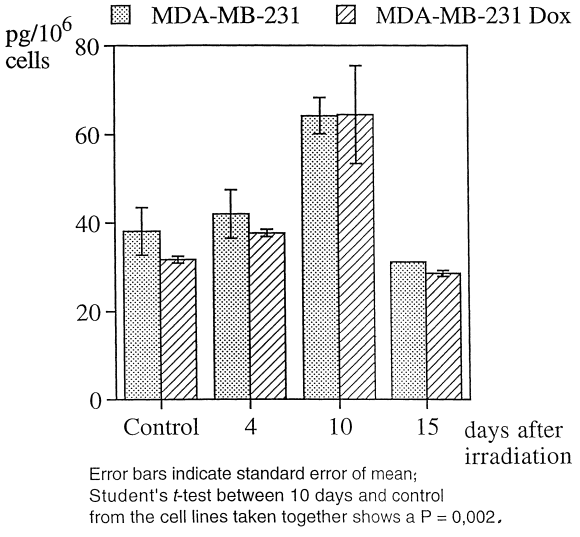


FIG. 13. The concentrations of BN-like peptide in non-irradiated cells (control) and 4, 10 and 15 days after 2 × 2 Gy irradiation.

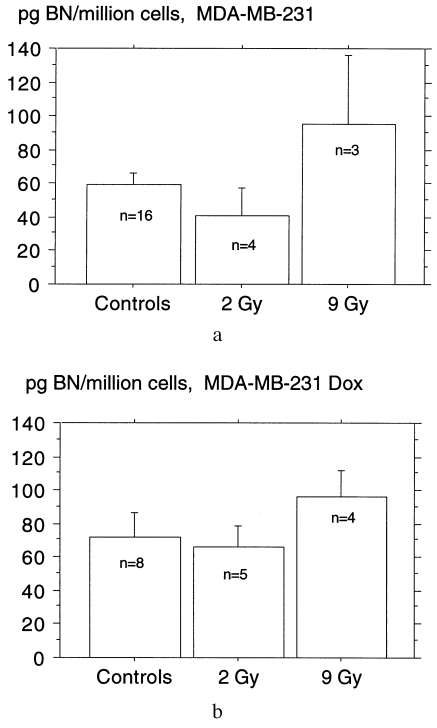


FIG. 14. The concentration of BN-like peptide in irradiated controls and following single doses of irradiation (2 and 9 Gy). a) MDA-MB-231 cell line, b) MDA-MB-231 doxorubicin resistant cell line.

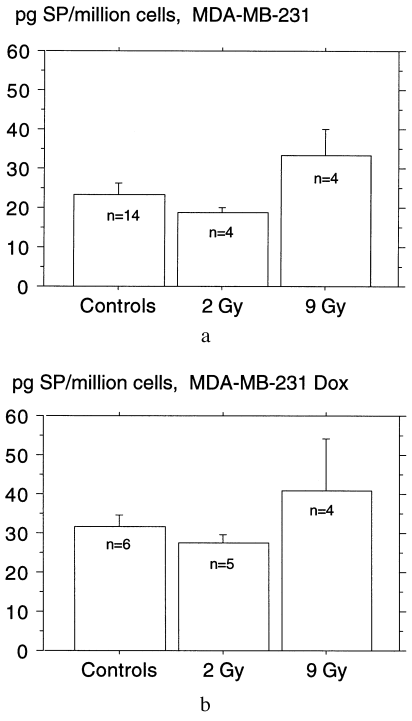


FIG. 16. The effect of single doses of irradiation (2 and 9 Gy) on the concentration of SP-like peptide in a) MDA-MB-231 cell line and b) MDA-MB-231 Dox (doxorubicin resistant) cell line.

phenomenon in common for various peptides, since irradiation could not increase the level of CGRP. The occurrence of a variability between different neuropeptides with respect to a changing pattern of immunoreaction has also previously been described for normal tissue (11,14).

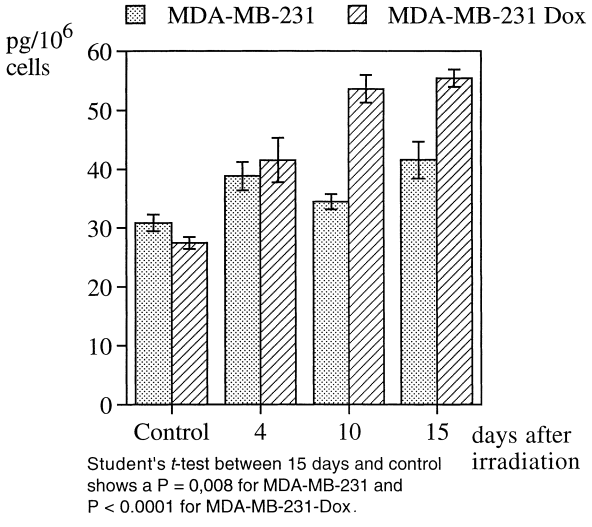


FIG. 15. The figure shows the concentrations of SP-like peptide in controls (non-irradiated cells), and 4, 10 and 15 days after 2 × 2 Gy irradiation.

It is well-known that endocrine differentiation not only occurs in classical endocrine tumors but also in nonendocrine neoplasms (3). With respect to breast carcinoma, approximately one third of the tumors show neuroendocrine features, as demonstrated by staining for neuron specific enolase (34). Immunoreactivity for BN has also earlier been demonstrated in breast carcinoma cells (28,33) but not in the normal breast or in benign breast lesions. Our observations of BN/GRP-LI in the cell line studied are thus in accordance with these previous observations. However, crossreactivity with so far unknown peptides cannot completely be excluded. It is previously well known that BN-antisera may at least partially crossreact with substance P, as BN-antisera may contain antibodies directed towards the C-terminal dipeptide shared with substance P and its related peptides (6). Therefore, the BN-antiserum was regularly preabsorbed with substance P. As we found cellular reactions in the tumor cells, after staining with BN/GRP antiserum, preabsorbed in this way, we conclude that the neuropeptide detected is BN/GRP but not substance P. Furthermore, the BN-antisera used in our RIA analysis has been shown to display only $<0.002\%$ cross-reactivity with substance P. The finding that the pattern of time-related changes, with respect to BN/GRP was not similar to that of SP, also shows that the RIA analyses have depicted the pattern of a BN/GRP peptide that is separate from a SP-peptide.

With respect to SP, it can be of interest to stress that the increase in synthesis of this peptide was most pronounced in the doxorubicin resistant cells. It is well known that one of the major problems in the treatment of cancer is the occurrence of either intrinsic (de novo) or acquired resistance to cancer treatment by the tumors. Unicellular resistance mechanisms include increased drug efflux, as in the case of P-glycoprotein overexpression and gene amplification, elevated glutathione levels, decreased drug accumulation, and increased DNA repair, among many others, and finally result in failure of chemotherapy. Sublethal radiation injury might alter the amount of SP and/or BN/GRP synthesized by tumor cells, in such a way that the neuropeptide may contribute to the relapses seen following radiotherapy, due to autocrine stimulation of tumor cell proliferation by affecting DNA synthesis. Moreover, the increased neuropeptide level may not only have a critical role in the repair of radiation induced damage in the irradiated cells themselves but also serve as a paracrine factor for the proliferation of adjacent cancer cells after irradiation.

BN/GRP and SP have indeed been shown to be potent growth factors with regard to both normal and tumor cell lines (38). BN/GRP has a role in the initiation and progression of some tumors (2,4,7,13,15,30,31) and is involved in the control of cell proliferation and DNA synthesis including transcriptional activation of nuclear oncogenes, such as c-fos and c-mys (9,21,27,37,47). Inositol phospholipid hydrolysis and Ca^{2+} efflux in MCF-7 and T47D human breast cancer cells are also stimulated by BN suggesting a role in mitogenic signalling (35). Moreover, BN/GRP has been shown to stimulate proliferation of the cell lines used in the present study (8,32,45), and the antagonist RC-3095 has also been found to inhibit growth in some cancers (24,25,39,45,46). SP has been shown to act as a mitogen for human blood T-lymphocytes and stimulates proliferation of embryonic rat aortic smooth muscle cells. Thus, it seems plausible that SP and BN/GRP in certain situations can act as potent stimulators of tumor cell growth.

In conclusion, the present study demonstrates that irradiation not only affects expression in neuropeptide-synthesizing neurons in normal tissue (1) but also in neuropeptide-synthesizing tumor cell populations. The increase seen might secondarily lead to a stimulation of tumor cell proliferation and be one of the biological bases to the recurrences obtained as well as to the encountered resistance to chemotherapeutics seen after sublethal irradiation (26).

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