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1,25-DIHYDROXYVITAMIN D_3 INCREASES EPIDERMAL GROWTH FACTOR RECEPTOR GENE EXPRESSION IN BT-20 BREAST CARCINOMA CELLS

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In the breast cancer cell line BT-20 which displays a high number of Epidermal Growth Factor Receptors (EGF-R), we have previously observed that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) at low concentration (0.1 nM) significantly reduces the proliferation rate while it up-regulates the EGF binding capacity. The aim of the present investigation was to analyze EGF-R mRNA expression in 1,25-$(OH)_2D_3$ treated BT-20 cells. It is found that in cells treated for several days, the EGF-R mRNA levels are increased in relation to the dose from 0.01 to 1 nM. To investigate the time course of the response, cells received the drug only once and were harvested at different times. The data suggest that the stimulation of EGF-R mRNA expression is dose- and time-dependent. Therefore, the increased EGF binding capacity previously demonstrated can be related to the increase of EGF-R transcript levels.

Epidermal Growth Factor (EGF) is one of the endogenous agents, hormones and growth factors, acting in concert in the regulation of mammary gland development and function. It induces a growth stimulating effect which seems to depend on the degree of differentiation of the cells (1, 2). Its activity is mediated by a transmembrane receptor protein (EGF-R) which has been identified as a glycoprotein of 170,000 Mr.

The EGF-R gene is located on chromosome 7 in human (3). According to the type of tissue it is differently regulated : by growth factors, steroids, vitamins, tumor necrosis factor and EGF itself. In normal breast, its levels of expression vary with the physiological endocrine states such as cycling, gestating or lactating (4). They are reduced in hypothyroid state and stimulated in vitro by retinoic acid (5, 6).

Data on EGF-R and its regulation in breast tumors were first obtained on cell lines (7). EGF-R content is usually expressed at the highest level in the less differentiated lines which are devoid of sex steroid receptors. In such cell lines, like MDA-468 and BT-20, the EGF-R gene is amplified and overexpressed (8, 9). In MDA-468, EGF itself up-regulates the level of EGF-R mRNA, and TGF-ß enhances the accumulation of EGF-R mRNA (10, 11). In T-47D cells, which are rich in progesterone receptors, treatment with progestin results in a dose-dependent increase in EGF-R mRNA (12).

Abbreviations: EGF, Epidermal Growth Factor; EGF-R, Epidermal Growth Factor Receptor; TGF-β, Transforming Growth Factor β; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

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While the dominant role of EGF on the breast epithelium is growth promotive and proliferative, the activity of 1,25-$(OH)_2D_3$ is essential for the maintenance of hormonally induced functional differentiation, membrane calcium transport (13) and milk protein synthesis (14). Indeed, mammary tumor cells are a target also for 1,25-$(OH)_2D_3$ (15). Previous in vitro studies in this laboratory showed that 1,25-$(OH)_2D_3$ reduces the proliferation rate of two human breast cancer cell lines BT-20 and MCF-7 (16) and stimulates the expression of a more differentiated morphology (17). Moreover, the EGF binding capacity of BT-20 cells is increased after 3 days of treatment with 1,25-$(OH)_2D_3$ (18) while it is reduced in MCF-7 within 24 h (19). The aim of the present investigation is to study the modifications of EGF-R gene expression in BT-20 cells treated by 1,25-$(OH)_2D_3$.

MATERIALS AND METHODS

Cells and cell culture. The BT-20 human mammary carcinoma cell line was supplied by Pr.W.L.McGUIRE (San Antonio, TX, USA). This cell line is characterized by the absence of estrogen (ER) and progesterone (PgR) receptors (20). As second source of cell material, the MCF-7 human mammary carcinoma cell line was used. This cell line, more differentiated than BT-20 cells, contains estrogen and progesterone receptors. Stock cultures of BT-20 and MCF-7 cells were grown in Dulbecco's modified Eagle Medium with Earle's salts, supplemented with L-Glutamin (2 mM) and heat inactivated fetal calf serum (5%), without antibiotics, at 37°C in humid atmosphere containing 5% CO_2. Culture medium and serum were obtained from Gibco (Flobio, France).
1,25-$(OH)_2D_3$ was a generous gift of Hoffmann-LaRoche (Basel, Switzerland). A stock solution was prepared at 10^{-3} M and stored under nitrogen at -20°C. Diluted solutions were used for addition to culture medium at the appropriate concentration. The ethanol volume added to the medium was 0.1 µl/ml.
For experiments, cells were seeded at 1-3 x10^4 cells per cm^2 in 100 mm-diameter Petri dishes. 1,25-$(OH)_2D_3$ treatment was started within 24 h when cell adhesion was complete. For the dose response experiments the steroid was added every day at the indicated concentrations over 3 days and for EGF-R mRNA studies the cells were harvested 6 h after the last steroid addition.
Binding assays were performed on cell monolayers at 24 h after the last 1,25-$(OH)_2D_3$ addition. The amount of EGF receptors present at the cell surface has been evaluated according to the methods previously described (18) by using EGF from murine origin labeled with ^{125}-Iodine.
For investigation of the time course of the response, cells received the drug only once at the indicated concentration and were harvested at successive times.

DNA extraction and Southern blot analysis. DNA extraction was performed according to the method of Maniatis et al, 1982 (21). DNA was obtained from the nuclei after lysis by 0.6% SDS, proteinase K digestion, phenol (Appligene) and chloroform treatments. DNA aliquots (10 µg) were digested by EcoRI enzymes (Boehringer Mannheim, Meylan, France), subjected to horizontal electrophoresis in a 0.7% agarose gel, and blot-transferred onto nylon filters (Hybond N^+, Amersham, England).

RNA extraction and Northern blot analysis. RNA extraction was performed according to the technique described by Chomczynski and Sacchi (22). Poly(A^+) mRNA was prepared by affinity chromatography with oligo(dT)-cellulose as described by Maniatis et al, 1982 (21). 10 µg of total RNA or 5 µg to 10 µg of poly (A^+) mRNA were analyzed by electrophoresis through a formaldehyde denaturing agarose gel (1%) and transferred to nylon filters (Genofit).

Probes, labeling and hybridization. A plasmid containing an insert (1.3 kb, EcoRI) corresponding to EGF-R transmembrane and protein kinase domains was kindly provided by Dr.Mark HUGHES (Baylor College of Medicine, Houston, USA). The probe has complete homology with the EGF-R cDNA sequence first published by Ullrich et al, 1984 (23). It was

labeled by random priming in the presence of (α-^{32}P) dCTP (Amersham) to a specific activity of 10^9 dpm/µg.
Hybridizations for DNA and RNA were performed according to the Rapid Hybridization System by Amersham.
The filters were exposed to Hyperfilm-MP (Amersham) at -80°C. Blot area and density on autoradiographic printing were assessed by a laser densitometer.
To assess the homogeneity of DNA loading, the filters were rehybridized with a GAPDH probe, and to check the integrity and the homogeneity of RNA loading, Northern filters were rehybridized with an 18S rRNA plasmid.

RESULTS

High molecular-weight DNA was extracted from the BT-20 and MCF-7 tumor cells, digested by EcoRI and hybridized with the EGF-R cDNA probe as indicated in Methods.
Figure 1A shows the hybridized EGF-R gene as a 4.8 kb band. As previously described (9), the BT-20 cell signal is amplified by 10 times over the level observed in MCF-7 cells. No evidence of gene rearrangement could be observed in the present experimental conditions. Rehybridization of the filter with a GAPDH probe confirmed that the amount of DNA loaded was equivalent in each lane (data not shown).
The Northern blot autoradiogram in Figure1B (1st lane) shows that in BT-20 cells the EGF-R probe hybridizes with 2 mRNA species, the major one being 10 and the other 5.6 kb size. The ratio of their density is approximately 4/1. EGF-R transcripts could not be detected in MCF-7 samples, even when 10 µg of poly (A^+) RNA were loaded and when the autoradiographic exposure was prolonged (Figure 1B 2nd lane). They have not been detected in these cells under basal conditions but only in experimental assays where they are up-regulated (24).
The ability of 1,25-$(OH)_2D_3$ to influence EGF-R expression in BT-20 cells was examined. Figure 2 (lanes 2 to 5) shows that in cells treated for 3 days, and harvested 6 h after the last steroid addition, the level of expression of both EGF-R species increases in relation to the dose

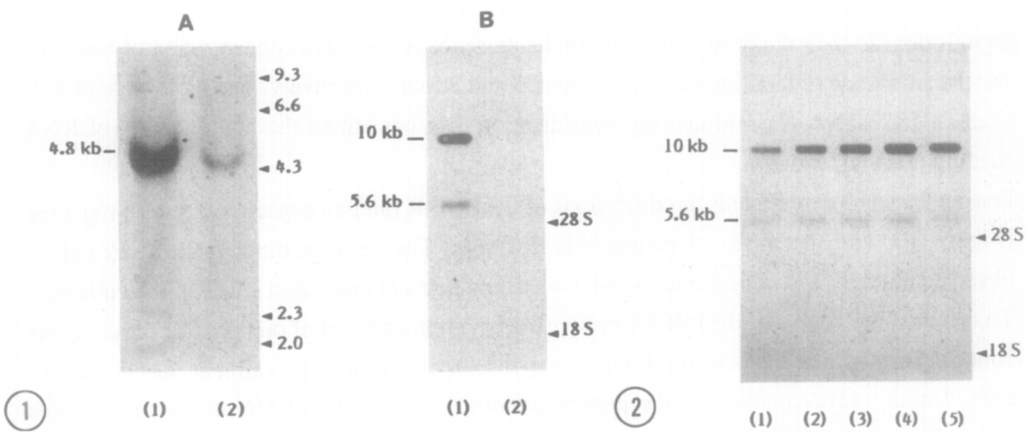
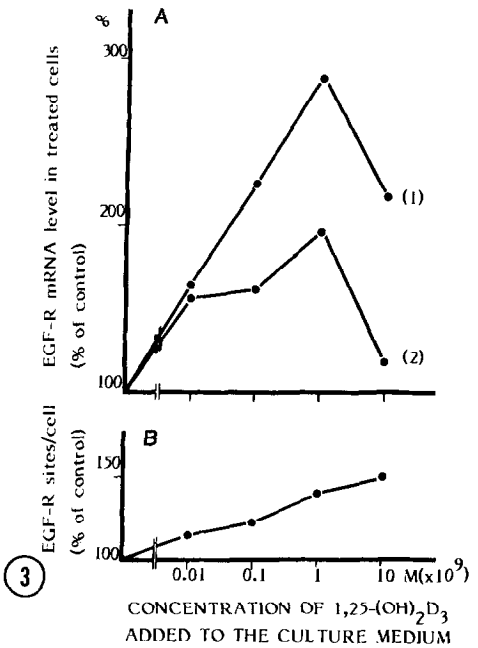


Figure 1A . Southern blot analysis of 10 µg of DNA from BT-20 cells (Lane1) and MCF-7 cells (Lane2) probed with EGF-R cDNA as described in Materials and Methods.
Figure 1B . Northern blot analysis of 10 µg of poly(A^+) mRNA from BT-20 cells (Lane1) and MCF-7 cells (Lane2) hybridized with the same probe as above.

Figure 2 . Northern blot analysis of BT-20 cell EGF-R transcripts. Lane 1: control cells. Lane 2,3,4,5 : cells treated for 3 days with 1,25-$(OH)_2D_3$ at 0.01, 0.1, 1, 10 nM concentrations and harvested at 6 h after the last hormone addition. All lanes contained 10 µg total RNA.



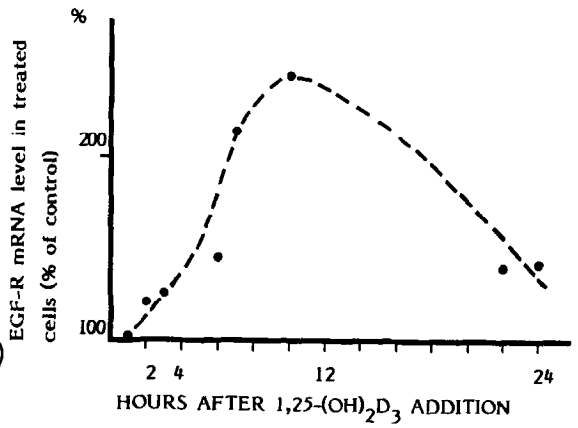


Figure 3 . Effect of increasing concentrations of 1,25-$(OH)_2D_3$ on EGF-R mRNA and EGF binding capacity. BT-20 cells were treated for 3 days and harvested as indicated in Methods. (A) Intensity of the 10 kb (1) and 5.6 kb (2) EGF-R mRNA signals in treated cells expressed as percent of mRNA level in control. (B) Number of binding sites in treated/control cells.

Figure 4 . Time course of the effects of 1,25-$(OH)_2D_3$ on EGF-R 10 kb mRNA level. Cells were harvested at the indicated time after hormone addition (zero time). They received 1 nM of hormone. The relative amount of mRNA was evaluated by density scanning of Northern blot autoradiogram.

of 1,25-$(OH)_2D_3$ which had been added to the cell culture medium. This effect is detectable for 0.01 nM and maximum for 1 nM concentration of the steroid, but at 10 nM the stimulatory effect was lower than at 1 nM. The data in Figure 3A clearly indicate that both 10 and 5.6 kb signals display their maximum intensity in the presence of the same concentration of hormone but the amplitude of their increase is different, 3 and 2 fold respectively. The blots were probed with an 18S rRNA plasmid and the hybridization data confirmed the homogeneity of RNA loading (data not shown).

During the same experiments, modifications of the EGF-R binding capacity were also registred (Figure 3B) and the mode of response is different. The peak in binding sites per cell is observed at the 10 nM concentration and it does not represent more than 150% of the control.

To test the time course of the mRNA response after administration of one dose of the drug, the concentration which leads to the highest response after prolonged treatment was chosen (1 nM). Under these conditions, the enhancement of the 10 kb mRNA message becomes detectable at 2-3 h (Figure 4). It is progressive at 4 and 10 h where it reaches 120 and 260% of the control respectively.

DISCUSSION

BT-20 cells display a high number of EGF receptor binding sites which is related to amplification and overexpression of the EGF-R gene (9). The present data demonstrate that the

level of expression of EGF-R mRNA can be modulated by 1,25-$(OH)_2D_3$ in relation to the dose which has been given to the cells. They are consistent with a previous report from this laboratory which had shown that treatment of these cells with 1,25-$(OH)_2D_3$ enhances the EGF binding capacity (18). Two RNA messengers of 10 and 5.6 kb size are detected, which are identical to those observed in normal placental tissue (25). Both are sensitive to concentrations of 1,25-$(OH)_2D_3$ as low as 0.01 nM. Both display a peak response for a 1 nM concentration and the 10 and 5.6 kb messengers are increased by 3 and 2 fold respectively in cells treated for 3 days and harvested at 6 h after the last 1,25-$(OH)_2D_3$ addition. The significance of these 2 messengers is not known. They are probably not related to the 2 types of binding sites defined by their respective dissociation constant since the ratio high vs low affinity number of sites is 1/1.5 and both are increased to the same extent in the treated cells (18).

Taken together, these data indicate that the increased EGF binding capacity described in BT-20 cells treated by 1,25-$(OH)_2D_3$ is associated to an increase of EGF-R mRNA which might be related either to an effect of the hormone on mRNA stability or to the stimulation -direct or indirect- of the gene transcription or both.

The regulation of EGF binding capacity by 1,25-$(OH)_2D_3$ treatment has been recently described on several human breast cancer cell lines by Koga et al (19). These authors did not detect any significant change in BT-20 cells and we suggest that this apparent discrepancy could be due to different culture conditions and a very short term 1,25-$(OH)_2D_3$ treatment. They investigated also the effects of the same treatment on the cell line T-47D and MCF-7, which are sex steroid responsive and display a low level of EGF-R : instead of an increase, they observed a rapid and acute decrease in the ^{125}I-EGF binding capacity. Such opposite effects of 1,25-$(OH)_2D_3$ on EGF-R in BT-20 cells and MCF-7 contrast with the reduction of the proliferation rate which is induced by this hormone on both cell lines to a similar extent (16).

All these effects of 1,25-$(OH)_2D_3$ are thought to be mediated in target cells by a nuclear receptor which belongs to the gene superfamily of steroid, thyroid and retinoid acid receptors (26). Binding of the hormone receptor complex to DNA regulatory sites results in control of transcription and translation of genes which are not yet all identified (26). 1,25-$(OH)_2D_3$ itself directly modulates the transcription of a number of genes which encode proteins that are specific of the cell differentiated functions (26). When applied to malignant cells, the return to a more differentiated stage is frequently associated with a reduction of proliferation (27). We have observed this association of events in BT-20 and MCF-7 cells treated by 1,25-$(OH)_2D_3$ for several days: the proliferation rate of both cell lines is decreased in the presence of low concentrations of 1,25-$(OH)_2D_3$ (16). The BT-20 cells, which do not express the morphological characteristics of epithelial glandular cells when grown in standard conditions, express again epithelial type cell junctions and glandular morphological characteristics when they are grown in the presence of 1,25-$(OH)_2D_3$ (17). Interestingly, this type of response is common to another differentiating agent, retinoic acid (28). The up-regulation of EGF-R mRNA by differentiating agents seems to be restricted to these undifferentiated malignant cells which do not respond to EGF by an increased proliferation.

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REFERENCES

1. Taketani, Y.,and Oka,T. (1983) Endocrinology 113,871-877.
2. Imagawa, W., Tomooka, Y., Hamamoto, S., and Nandi, S. (1985) Endocrinology 116,1514-1524.
3. Spurr, N.K., Solomon, E., Jansson, M., Sheer, D., Goodfellow, P.N., Bodmer, W.F., and Vennstrom, B. (1984) EMBO J 3,159-163.
4. Edery, M., Pang, K., Larson, L., Colosi, T., and Nandi, S. (1985) Endocrinology 117,405-411.
5. Vonderhaar, B.K., Tang, E., Lyster, R.R., and Nascimento, M.C.S. (1986) Endocrinology 119,580-585.
6. Komura, H., Wakimoto, H., Chu-Fung, C., Terakawa, N., Aono, T., Tanizawa, O., and Matsumoto, K. (1986) Endocrinology 118,1530-1536.
7. Imai, Y., Leung, C.K.H., Friesen, H.G., and Shiu, R.P.C. (1982) Cancer Res 42,4394-4398.
8. Filmus, J., Pollak, M.N., Cailleau, R., and Buick, R.N. (1985) Biochem Biophys Res Commun 128,898-905.
9. Lebeau, J., and Goubin, G. (1987) Int J Cancer 40,189-191.
10. Bjorge, J.D., Paterson, A.J., and Kudlow, J.E. (1989) J Biol Chem 264,4021-4027.
11. Fernandez-Pol, J.A., Klos, D.J., Hamilton, P.D., and Talkad, V.D. (1987) Cancer Res 47,4260-4265.
12. Murphy, L.C., Murphy, L.J., and Shiu, R.P.C. (1988) Biochem Biophys Res Commun 150,192-196.
13. Mezzetti, G., Monti, M.G., Pernecco Casolo, L., Piccinini, G., and Moruzzi, M.S. (1988) Endocrinology 122,389-394.
14. Bhattacharjee, M., Wientroub, S., and Vonderhaar, B.K. (1987) Endocrinology 121,865-874.
15. Findlay, D.M., Michelangeli, V.P., Eisman, J.A., Frampton, R.J., Moseley, J.M., MacIntyre, I., Whitehead, R., and Martin, T.J. (1980) Cancer Res 40:4764-4767.
16. Chouvet, C., Vicard, E., Devonec, M., and Saez, S. (1986) J Steroid Biochem 24,373-376.
17. Frappart, L., Falette, N., Lefebvre, M.F., Bremond, A., Vauzelle, J.L., and Saez S. (1989) Differentiation 40,63-69.
18. Falette, N., Frappart, L., Lefebvre, M.F., and Saez, S. (1989) Mol Cell Endocrinol 63,189-198.
19. Koga, M., Eisman, J.A., and Sutherland, R.L. (1988) Cancer Res 48,2734-2739.
20. Horwitz, K.B., Zava, D.T., Thilagar, A.K., Jensen, E.M., and McGuire, W.L. (1978) Cancer Res 38,2434-2437.
21. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NewYork.
22. Chomczynski, P., and Sacchi, N. (1987) Anal Biochem 162,156-159.
23. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., and Seeburg, P.H. (1984) Nature 309,418-425.
24. Lee, C.S.L., Koga, M., and Sutherland, R.L. (1989) Biochem Biophys Res Commun 162,415-421.
25. Chen, C.F., Kurachi, H., Fujita, Y., Terakawa, N., Miyake, A., and Tanizawa, O. (1988) J Clin Endocrinol Metab 67,1171-1177.
26. Haussler, M.R., Mangelsdorf, D.J., Komm, B.S., Terpening, C.M., Yamaoka, K., Allegretto, E.A., Baker, A.R., Shine, J., McDonnell, D.P., Hughes, M., Weige, N.L., O'Malley, B.W., and Pike, J.W. (1988) Recent progress in hormone research. Acad Press, vol 44:263-297.
27. Sidell, N. (1982) J Clin Invest 68,589-593.
28. Nakajima, M., Lotan, D., Baig, M.M., Carralero, R.M., Wood, W.R., Hendrix, M.J.C., and Lotan, R. (1989) Cancer Res 49,1698-1706.