J. Steroid Biochem. Molec. Biol. Vol. 43, No. 1–3, pp 111–115, 1992
Printed in Great Britain

0960-0760/92 $5.00 + 0.00
Pergamon Press Ltd

DIRECT STIMULATION BY ESTROGEN OF GROWTH FACTOR SIGNAL TRANSDUCTION PATHWAYS IN HUMAN BREAST CANCER CELLS

BART VAN DER BURG,* ROLF P. DE GROOT, LINDA ISBRÜCKER, WIEBE KRUIJER and SIEGFRIED W. DE LAAT

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Summary—Estrogen is thought to stimulate the proliferation of human breast tumors indirectly, through induced production of autocrine polypeptide growth factors. Constitutive production of such growth factors would lead to the loss of 17β-estradiol (E2)-dependence that is associated with progression of the disease. Our data, however, do not support this hypothesis and suggest that hormone-dependent breast tumor cell lines like MCF7 do not react to the growth factors which they produce. Moreover, we provide evidence that E2 directly stimulates proliferation by inducing, like many growth factors, the c-*fos* proto-oncogene. E2 by itself, however, is poorly mitogenic. This may be caused by the lack of induction of genes from the *jun* family, whose gene products are necessary for dimerization with the c-*fos* encoded protein, leading to an important step in growth factor signalling pathways; stimulation of TPA responsive element (TRE)-dependent transcriptional activity. In combination with insulin-like growth factors, efficient inducers of c-*jun* in these cells, E2 synergistically stimulates proliferation and TRE-activity. Constitutive TRE-activation may lead to loss of E2-dependence.

INTRODUCTION

Part of human breast tumor are 17β-estradiol (E2)-dependent and regress upon hormonal therapy. Evidence has been presented that growth stimulation by E2 is an indirect process mediated by autocrine acting E2-induced polypeptide growth factors, such as insulin-like growth factors (IGFs) and transforming growth factor α (TGFα; reviewed in [1]). As this disease progresses, however, the response to hormonal therapy is often lost and regrowth of E2-independent tumors occurs. Autonomous production of E2-inducible growth factors has been proposed to be the cause of this loss of E2-dependence [1]. Our data, however, do not support the above model and suggest that E2 stimulates proliferation of breast tumors by more direct mechanisms.

DEFINED CULTURE CONDITIONS REVEAL STRICT DEPENDENCE OF MCF7 CELL PROLIFERATION NOT ONLY ON E2, BUT ALSO INSULIN OR IGFs

To shed light on the process leading to the loss of hormone-dependence we have studied the mechanism by which E2 and polypeptide growth factors stimulate proliferation of the human breast cancer cell line MCF7. For a detailed study of this mechanism it is essential to perform such studies without background mitogenic stimulation. To avoid many of the difficulties associated with culturing human breast cancer cells under strict serum-free conditions [2], we used media supplemented with serum in which growth factors were chemically inactivated and steroids are removed (referred to as DCC-SH-FCS; [3]). This serum was used to supplement a phenol red-free [4] culture medium, containing bovine serum albumin (BSA), selenite and transferrin. The thus obtained medium is a very suitable general-purpose medium, and when appropriate growth factors are added it supports proliferation of a large number of cell lines, including lines for which serum-free culture conditions have not been described ([5] and references therein). Autonomously growing tumor cell lines, such as the E2-independent breast tumor cell lines MDA-MB231, MDA-MB468, Hs578T and BT20 proliferate in this medium in the absence of added mitogens [6].

In contrast, as a result of this mitogen depletion the E2-dependent human breast cancer cell line MCF7 becomes quiescent in the

Proceedings of the Fourth International Congress on Hormones and Cancer, Amsterdam, The Netherlands, September 1991.

*To whom correspondence should be addressed.

G1/G0 phase of the cell cycle, from which it can be stimulated to enter the cell cycle by using defined mitogens [3]. This growth arrest is not observed in medium containing conventional serum that is treated only with dextran coated charcoal to remove endogenous steroids (DCC-FCS), since growth factors remain present causing background mitogenic activity [3]. Thus, under our conditions MCF7 cells displayed their strict hormone-dependence that has been established *in vivo* (in nude mice), under physiological conditions [7, 8].

Surprisingly, under these defined culture conditions E2 alone was a poor mitogen for the hormone-dependent breast cancer cell lines MCF7 and CG5 [3]. However, its mitogenic action was potentiated by the presence of either DCC-FCS, insulin or insulin-like growth factors (IGFs). In MCF7 cells, the synergistic effect with E2 was half-maximal at 3 ng/ml of insulin or at 5-times lower concentrations of IGF-I [9], which suggests involvement of the IGF-I receptor. At much higher concentrations insulin and related factors are fully mitogenic and, unlike growth factors like TGFα, EGF, FGF, PDGF, TGFβ, are able to bypass E2-requirement [3]. Therefore, IGFs seemed the prime candidates to serve an autocrine function.

E2-INDUCED PROLIFERATION OF MCF7 CELLS SEEMS NOT TO BE MEDIATED BY AUTOCRINE GROWTH FACTORS

We used several experimental approaches to test if IGFs are important autocrine mediators of E2-induced mitogenesis. In the first place we performed kinetic studies which showed that E2 stimulates DNA synthesis without a delay-period compared to IGFs, giving a first indication that no growth factor production is involved in E2-induced mitogenesis [10]. In addition it was found that IGF binding proteins [6] or antibodies [11] that efficiently blocked IGF-induced mitogenesis did not inhibit the E2-response of MCF7 cells. Rohlik *et al.* [12] reported blockade of E2-induced, but also basal, proliferation in DCC-FCS supplemented medium by an antibody that blocks IGF-I binding to its receptor. This, however, does not prove the existence of an E2-induced autocrine loop but rather confirms our data that show that proliferation of MCF7 cells depends on external IGFs or insulin (crossreacting with the IGF-I receptor). In the study of Rohlik *et al.* [12] the DCC-FCS is the source of these growth factors.

Next, we set out to investigate the production of biologically active IGFs by breast cancer cell lines. For this purpose, a bioassay for IGFs was developed [9, 13]. In this assay no activity was found in conditioned media of E2-treated MCF7 cells or E2-independent human breast cell lines, again suggesting a minor role of autocrine produced growth factors in the regulation of human breast cancer proliferation [6]. The large amount of immunoreactive IGF-I that has been reported to be present in conditioned medium of breast cancer cell lines [14] may in fact be IGF binding protein instead [15]. After efficient removal of these binding proteins, it was shown that only trace amounts of immunoreactive IGF-I can be detected in MCF7 conditioned medium [15, 16]. The mitogenic activity of highly concentrated conditioned medium [17, 18] seems to be caused by an activation of serum-derived growth factors e.g. by E2-induced proteolytic enzymes [6, 19], since these experiments cannot be repeated under conditions in which no externally added growth factors are present [6, 15]. Until now little conclusive evidence is available that demonstrates the existence of autocrine loops in breast cancer cells. In fact, recent results obtained in various laboratories also suggest that breast cancer cells generally do not react to the growth factors which they produce (reviewed in [20]).

PARACRINE REGULATION OF HORMONE-DEPENDENT BREAST CANCER

Besides E2, MCF7 cells depend on external IGFs for their proliferation (see above). Possibly the large amount of stromal cells often present in breast tumors may be an important source of IGFs, since human fibroblasts secrete biologically active IGFs [6], a process stimulated by PDGF [21]. The production of growth factors for stromal cells like PDGF [22–24] and TGFα [24–26] by the epithelial cells might lead to a mutual dependence of both cell types in the tumor. Since E2 stimulates production of both PDGF [23] and TGFα [25, 26] by breast cancer cells, it might be the major endocrine factor that is necessary to keep this process of mutual paracrine stimulation going [6]. In addition, endocrine acting IGFs may also be important in stimulating proliferation of MCF7 cells. The

reaction to external growth factors of the breast cancer cells may be changed by production of binding proteins [6] and by proteolytic enzymes.

THE ROLE OF NUCLEAR PROTO-ONCOGENES IN THE E2-INDUCED PROLIFERATION OF MCF7 CELLS

Nuclear proto-oncogenes like c-*fos* and c-*myc* are a primary target of the signal transduction that leads from growth factor receptors to the nucleus (reviewed in [27]). The products of these genes are among the first to be induced after mitogenic stimulation of cells and are involved in transforming the incoming signal into a change in gene expression. The expression of these transcription factors is rapidly down-regulated and constitutive expression can lead to cellular transformation.

The product of c-*fos* (Fos) can only bind to DNA after formation of heterodimers with other transcription factors. Together with proteins belonging to the Jun family it forms AP1, a dimeric transcription factor [28–32]. This interaction results in a greatly enhanced affinity for the AP1 target sequence, the TPA-responsive element (TRE), and a stimulation of TRE-dependent transcriptional activity [28, 32, 33]. The gene coding for Jun (*jun*) is also growth factor inducible [33–36]. Induction of AP1 and TRE activity is thought to be a critical step in the onset of cellular proliferation and transformation by polypeptide growth factors (reviewed in [27]). In this process c-*fos* expression is essential since suppression of expression by antisense RNA abolishes the TRE-dependent induction of genes, and cellular proliferation and transformation.

We and others have observed a rapid induction of the growth-related proto-oncogenes c-*fos* [10] and c-*myc* [10, 37] upon E2-treatment of MCF7 cells. This induction is a direct effect, independent of induced proteins, showing that no growth factor production is involved in E2-induced expression of these proto-oncogenes [10, 37]. In spite of the induction of the c-*fos* proto-oncogene, E2 does not induce c-*jun* and hardly induces TRE activity [38]. In contrast, insulin at high concentrations (10 μg/ml) efficiently induces both c-*fos* and c-*jun* and TRE activity in MCF7 cells. At concentrations of insulin which are suboptimal for induction of proliferation, also suboptimal TRE activation is observed [38]. Interestingly, at these concentrations of insulin E2 synergistically stimulates both proliferation and TRE activity. Under these conditions the TRE induction is probably caused by insulin-induced c-*jun* in combination with E2-induced c-*fos*. In MCF7 cells TRE activation can indeed be induced by the combined action of c-*jun* and c-*fos* (van der Burg *et al.*, unpublished).

Recently, Weisz *et al.* [39] reported efficient induction of TRE activity in MCF7 cells by E2 alone. They assumed that this is due to the induction of not only c-*fos*, but also c-*jun* in these cells. Our data, however, show that E2 does not induce c-*jun* in MCF7 [38]. The stimulation of TRE activity by E2 was shown in DCC-FCS containing medium [39], and is probably caused by c-*jun* induction by the serum-derived IGFs, in combination with E2-induced c-*fos*. In DCC-FCS we also find induction of TRE activity by E2 alone (van der Burg *et al.*, unpublished).

In conclusion, we found that TRE-dependent transcriptional activity is induced when MCF7 cells are optimally stimulated to proliferate, either by insulin at high concentrations or the synergistic combination of low concentrations of insulin together with the steroid hormone E2. In addition, this activity is also efficiently induced by the phorbol ester TPA, a compound that leads to growth arrest in these cells, probably by inducing differentiation [40, 41]. Therefore, TRE activity may be obligatory, but no guarantee for the induction of cellular proliferation. Also in other cells it probably plays a role in induction of differentiation [42]. A differential induction of other early response genes or differences in post-translational modifications may influence the control of important growth regulatory genes by the mitogens and differentiation inducing agents.

CONCLUSION

Our results show that by inducing c-*fos* expression E2 is able to directly interact with nuclear events that are also induced by polypeptide growth factors and that represent a critical step in the induction of cellular proliferation and tumorigenesis [27]. This implicates that growth factor secretion may not be an essential part of this mitogenic pathway elicited by E2, as was initially proposed [1]. This is in line with recent data which suggest that MCF7 cells do not react to the growth factors which they secrete (reviewed in [20]). Stimulation of TRE activity in cells by TPA treatment or

transfection with activated *ras* oncogenes (reviewed in [43]), results in growth factor secretion [44–46]. Therefore growth factor secretion by MCF7 cells may be a consequence rather than a cause of growth stimulation [20, 38]. Interestingly, when MCF7 cells are transfected with the potent v-Ha-*ras* oncogene, a loss of E2-dependence concomitant with elevated growth factor secretion has been observed [47, 48]. Possibly, not this elevated growth factor secretion but the direct activation of TRE activity is the cause of the autonomous proliferation. It is tempting to speculate that in human breast tumors a similar process underlies the conversion towards hormone-independence that is associated with progression.

Acknowledgement—This work was supported by the Netherlands Cancer Foundation.

REFERENCES

1. Dickson R. B. and Lippman M. E.: Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocrine Rev.* **8** (1987) 29–43.
2. Barnes D. W.: Serum-free cell culture of MCF7 human mammary carcinoma. In *Methods for Serum-free Culture of Cells of the Endocrine Systems* (Edited by D. W. Barnes, D. A. Sirbasku and G. H. Sato). Alan R. Liss, New York (1984) pp. 201–216.
3. Van der Burg B., Rutteman G. R.,. Blankenstein M. A., de Laat S. W. and van Zoelen E. J. J.. Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: synergistic action of insulin and estrogen. *J. Cell Physiol.* **123** (1988) 101–108
4. Berthois Y., Katzenellenbogen J. A. and Kazenellenbogen B. S.: Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 2496–2500.
5. Van der Burg B., van Selm-Miltenburg A. J. P., van Maurik P., Rutteman G. R., Misdorp W., de Laat S. W. and van Zoelen E. J. J . Isolation of autonomously growing dog mammary tumor cell lines cultured in medium supplemented with serum treated to inactivate growth factors. *J. Natn. Cancer. Inst.* **81** (1989) 1545–1551.
6. Van der Burg B., Isbrücker I., van Selm-Miltenburg A. J. P., de Laat S. W. and van Zoelen E. J. J.: The role of estrogen-induced insulin-like growth factors in the proliferation of human breast cancer cells. *Cancer Res.* **50** (1990) 7770–7774.
7. Shafie S. M. and Liotta L. A.: Formation of metastasis by human breast carcinoma cells (MCF-7) nude mice. *Cancer Lett.* **11** (1980) 81–87.
8. Soule H. D. and McGrath C. M.: Estrogen responsive proliferation of clonal human breast carcinoma cells in athymic mice. *Cancer Lett.* **10** (1980) 177–189.
9. Van Zoelen E. J. J., Ward-van Oostwaard Th. M. J., Nieuwland R., van der Burg B., van den Eijnden-van Raaij A. J. M., Mummery C. L. and de Laat S. W.: Identification and characterization of polypeptide growth factors secreted by murine embryonal carcinoma cells. *Dev. Biol.* **133** (1989) 272–283.
10. Van der Burg B., van Selm-Miltenburg A. J. P., de Laat S. W. and van Zoelen E. J. J.: Direct effect of estrogen on c-*fos* and c-*myc* proto-oncogene expression and cellular proliferation in human breast cancer cells. *Molec. Cell. Endocr.* **64** (1989) 223–228.
11. Arteaga C. L. and Osborne C. K.: Growth inhibition of human breast cancer cells *in vitro* with an antibody against the type I somatomedin receptor. *Cancer Res.* **49** (1989) 6237–6241.
12. Rohlik Q. T., Adams D., Kull F C. and Jacobs S.. An antibody to the receptor of insulin-like growth factor I inhibits the growth of MCF-7 cells in tissue culture. *Biochem. Biophys. Res Commun.* **149** (1987) 276–281
13. Van Zoelen E J. J., Koorneef I., Holthuis J C. M., Ward-van Oostwaard T. M. J., Feijen A., De Poorter T. L., Mummery C. L. and Van Buul-Offers S. C.. Production of insulin-like growth factors, platelet-derived growth factor and transforming growth factors and their role in the density dependent growth regulation of a differentiated embryonal carcinoma cell line. *Endocrinology* **124** (1989) 2029–2041.
14. Huff K. K., Kaufman D., Gabbay K. H , Spencer E. M., Lippman M. E. and Dickson R. B.: Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells *Cancer Res.* **46** (1986) 4613–4619.
15. Ahmed S. R., Manni A., Gray G. and Hammond J. M.: Characterization and hormonal regulation of radioimmunoassayable IGF-I (Insulin-like growth factor I) like activity and IGF-binding proteins secreted by human breast cancer cells *Anticancer Res.* **10** (1990) 1217–1224.
16. Karey K P. and Sirbasku D. A.: Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17a-estradiol. *Cancer Res.* **48** (1988) 4083–4092.
17. Vignon F., Derocq D., Chambon M. and Rochefort H.. Estrogen-induced proteins secreted by MCF-7 human breast cancer cells stimulate their proliferation. *C. R. Acad. Sci. Paris* **296** (1983) 151–156.
18. Dickson R. B., Huff K. K., Spencer E. M. and Lippman M. E.· Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. *Endocrinology* **118** (1985) 138–142.
19. Vignon F., Capony F., Chambon M., Freiss G., Garcia M. and Rochefort H.: Autocrine growth stimulation of the MCF7 breast cancer cells by the estrogen-regulated 52K protein. *Endocrinology* **118** (1986) 1537–1545.
20. Van der Burg B. and de Laat S. W.: Molecular action of steroids and growth factors in human breast tumor cells In *Recent Advances in Cell To Cell Signals in Plants and Animals* (Edited by V. Neuhoff and J. Friend). Springer Verlag, Berlin (1991) pp. 256–287.
21. Clemmons D. R., Underwood L. E. and van Wyk J. J.: Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. *J. Clin. Invest.* **67** (1981) 10–19.
22. Rozengurt E. and Sinnett-Smith J.: Production of PDGF-like growth factor by breast cancer cell lines. *Int. J. Cancer* **36** (1985) 247–253.
23. Bronzert D. A., Pantazis P., Antoniades H. N., Kasid A., Davidson N., Dickson R. B. and Lippman M. E.: Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 5763–5767.
24. Peres R., Betsholtz C., Westermark B. and Heldin C.-H.: Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. *Cancer Res.* **47** (1987) 3425–3429.
25. Bates S., Manaway M. E., Lippman M. E. and Dickson R. B.: Characterization of estrogen responsive transforming activity in human breast cancer cell lines. *Cancer Res.* **46** (1986) 1707–1713.

26. Bates S. E., Davidson N. E., Valverius E. M. and Lippman M. E.: Expression of transforming growth factorα and its messenger ribonucleic acid in human breast cancer: its regulation by estrogen and its possible functional significance. *Molec. Endocr.* **2** (1988) 543–555.
27. Herrlich P. and Ponta H.: "Nuclear" oncogenes convert extracellular stimuli into changes in the genetic program. *TIG* **5** (1989) 112–116.
28. Chiu R., Boyle W. J., Meek J., Smeal T., Hunter T. and Karin M.: The c-*fos* protein interacts with c-*jun*/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* **54** (1988) 541–552.
29. Halazonetis T. D., Georgopoulos K., Greenberg M. E. and Leder P.: c-*jun* dimerizes with itself and with c-*fos*, forming complexes of different DNA binding affinities. *Cell* **55** (1988) 917–924.
30. Nakabeppu Y., Ryder K. and Nathans D.: DNA binding activities of three murine Jun proteins: stimulation by Fos. *Cell* **55** (1988) 907–915.
31. Rauscher F. J., Sambucetti L. C., Curran T., Distel R. J. and Spiegelman B. M.: Common DNA binding site for Fos protein complexes and transcription factor AP-1. *Cell* **52** (1988) 471–480.
32. Sassone-Corsi P., Lamph W. W., Kamps M. and Verma I. M.: *fos*-associated cellular p39 is related to nuclear transcription factor AP-1. *Cell* **54** (1988) 553–560.
33. Lamph W. W., Wamsley P., Sassone-Corsi P. and Verma I. M.: Induction of proto-oncogene JUN/AP-1 by serum and TPA. *Nature* **334** (1988) 629–631.
34. Quantin B. and Breathnach R.: Epidermal growth factor stimulates transcription of the c-*jun* proto-oncogene in rat fibroblasts. *Nature* **334** (1988) 538–539.
35. Ryder K. and Nathans D.: Induction of proto-oncogene c-*jun* by serum growth factors. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 8464–8467.
36. Ryseck R.-P., Hirai S. I., Yaniv M. and Bravo R.: Transcriptional activation of c-*jun* during the GI/G0 transition in mouse fibroblasts. *Nature* **334** (1988) 535–537.
37. Dubik D. and Shiu R. P. C.: Transcriptional regulation of c-*myc* oncogene expression in hormone-responsive human breast cancer cells. *J. Biol. Chem.* **236** (1988) 12705–12708.
38. Van der Burg B., de Groot R. P., Isbrücker L., Kruijer W. and de Laat S. W.: Stimulation of TRE activity by a cooperative action of insulin and estrogen in human breast cancer cells. *Molec. Endocr.* **4** (1990) 1720–1726.
39. Weisz A., Cicatiello L., Persico E., Scalona M. and Bresciani F.: Estrogen stimulates transcription of c-*jun* protooncogene. *Molec. Endocr.* **4** (1990) 1041–1050.
40. Abe M. and Kufe D.: Effects of maturational agents on expression and secretion of two partially characterized high molecular weight milk-related glycoproteins in MCF-7 breast carcinoma cells. *J. Cell Physiol.* **126** (1986) 126–132.
41. Valette A., Gas N., Jozan S., Dupont M. A. and Bayard F.: Influence of 12-*o*-tetradecaoylphorbol-13-acetate on proliferation and maturation of human breast carcinoma cells (MCF-7): relationship to cell cycle events. *Cancer Res.* **47** (1987) 1615–1620.
42. Bartel D. P., Sheng M., Lau L. F. and Greenberg M. E.: Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. *Genes Dev.* **3** (1989) 304–313.
43. Imler J. L. and Wasylyk B.: A composite transcription factor implicated in abnormal growth control. *Prog. Growth Factor Res.* **1** (1989) 69–77.
44. Bjorge J. D., Paterson A. J. and Kudlow J. E.: Phorbol ester or epidermal growth factor (EGF) stimulates the concurrent accumulation of mRNA for the EGF receptor and its ligand transforming growth factor-α in a breast cancer cell line. *J. Biol. Chem.* **264** (1989) 4021–4027.
45. Pittelkow M. R., Lindquist P. B., Abraham R. T., Graves R. T., Derynck R. and Coffey R. J.: Induction of transforming growth factor-α expression in human keratinocytes by phorbol esters. *J. Biol. Chem.* **264** (1989) 5164–5171.
46. Colamonici O. R., Trepel J. B., Vidal C. A. and Neckers L. M.: Phorbol ester induces c-*sis* gene transcription in stem cell line K-562. *Molec. Cell. Biol.* **6** (1986) 1847–1850.
47. Dickson R. B., Kasid A., Huff K. K., Bates S. E., Knabbe C., Bronzert D., Gelmann E. P. and Lippman M. E.: Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17β-estradiol or v-Ha-*ras* oncogene. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987) 837–841.
48. Kasid A., Knabbe C. and Lippman M. E.: Effect of v-Ha-*ras* oncogene transfection on estrogen-independent tumorigenicity of estrogen-dependent human breast cancer cells. *Cancer Res.* **47** (1987) 5733–5738.