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LIMB MESENCHYMAL CELLS INHIBITED FROM UNDERGOING CARTILAGE DIFFERENTIATION BY A TUMOR PROMOTING PHORBOL ESTER MAINTAIN EXPRESSION OF THE HOMEOBOX-CONTAINING GENE *MSX1* AND FAIL TO EXHIBIT GAP JUNCTIONAL COMMUNICATION

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SUMMARY: Tumor promoting phorbol esters are potent inhibitors of the chondrogenic differentiation of limb mesenchymal cells, but the mechanism by which these agents elicit their antichondrogenic effect is unknown. Here we report that limb mesenchymal cells inhibited from undergoing chondrogenesis by a tumor promoting phorbol ester exhibit deregulated expression of the homeobox-containing gene *Msx1*, a gene implicated in suppressing differentiation of limb mesenchymal cells, and fail to exhibit the extensive gap junctional intercellular communication that normally occurs at the onset of chondrogenesis. These results suggest that tumor promoting activity of phorbol esters may relate to their ability to modulate the expression of regulatory genes involved in controlling terminal differentiation, as well as to their ability to disrupt the intercellular communication involved in initiating the differentiated phenotype of cells.

Phorbol esters such as TPA are potent tumor promoters which activate protein kinase C [1]. Tumor promoting phorbol esters modulate the differentiation of a variety of cell types, and it has been suggested that one general mechanism of their action might be to modulate the expression of regulatory genes involved in controlling terminal differentiation [1,2]. In addition, phorbol esters and other tumor promoters inhibit gap junctional intercellular communication in a variety of cell types [3-6], and it has been suggested that their tumor promoting activity may be related to their ability to disrupt the intercellular communication required for normal tissue homeostasis and growth control [6].

Tumor promoting phorbol esters such as TPA are potent inhibitors of the chondrogenic differentiation of embryonic chick limb mesenchymal cells in vitro [7-10], but the mechanism by which these agents elicit their anti-chondrogenic effect is unknown. We have found that limb mesenchymal cells inhibited from undergoing chondrogenic differentiation by TPA exhibit

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ABBREVIATIONS: TPA: 12-O-tetradecanoyl phorbol 13-acetate; Cx43: connexin43; Hox: homeobox-containing; AER: apical ectodermal ridge.

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deregulated expression of the Hox gene *Msx1*, a gene that has been implicated in maintaining limb mesenchymal cells in a labile undifferentiated condition [see 11 for review], and also fail to exhibit the extensive gap junctional intercellular communication that normally occurs at the onset of limb cartilage differentiation when critical cell-cell interactions are occurring that are necessary to trigger cartilage differentiation [12]. These observations provide support for the hypotheses that the tumor promoting activity of phorbol esters may relate to their ability to modulate the expression of regulatory genes that are involved in controlling terminal differentiation, as well as to their ability to disrupt the intercellular communication that may be involved in initiating and/or maintaining the differentiated phenotype of cells.

METHODS

High density micromass cultures were prepared from the undifferentiated mesenchymal cells comprising the distal subridge region of stage 25 [13] embryonic chick wing buds as described [14], and cultured in medium containing 0.1 mM TPA or vehicle. Cartilage matrix deposition by the cultures was routinely monitored by staining with Alcian blue, pH 1.0 [14]. Gap junctional communication in control and TPA-treated cultures was assayed by the scrape-loading/dye transfer procedure of El-Fouly et al. [15] as described [12]. Briefly, cultures were incubated in a solution containing 0.05% of the gap junction-permeable dye lucifer yellow and the gap junction-impermeable dye rhodamine dextran, and incisions were made with a fine scalpel blade throughout the diameter of the cultures [12]. Transfer of lucifer yellow dye from incised cells to contiguous cells was allowed to proceed for 2 min, after which the cultures were fixed and the extent of dye transfer visualized by fluorescence microscopy [12]. Steady-state levels of mRNAs for the gap junction protein Cx43 and the Hox gene *Msx1* were determined by a modification of the cytoplasmic dot hybridization procedure of White and Bancroft [16] as previously described [17], except that RNA was extracted from the cytoplasmic extracts with phenol/chloroform as described by Leonard et al. [18]. Cx43- and *Msx1*- specific hybridization probes were prepared by the polymerase chain reaction as previously described [19,20]. Levels of hybridizable RNA sequences were quantified by densitometry as described [17]. The total poly(A)+ content of the extracted RNA was determined by the procedure of Harley [21].

RESULTS AND DISCUSSION

As shown in Fig. 1a,b, TPA at a concentration of 0.1 mM completely inhibits the accumulation of Alcian blue, pH 1.0-stainable cartilage matrix by micromass cultures prepared from the undifferentiated mesenchymal cells comprising the distal tip of stage 25 embryonic chick wing buds, confirming the results of several other investigators that phorbol esters inhibit limb cartilage differentiation [7-10].

To explore the possibility that TPA might affect the activity of genes involved in regulating terminal chondrogenic differentiation, we examined the effect of TPA on the expression of the Hox gene *Msx1* by limb mesenchymal cell micromass cultures. *Msx1* (formerly called *GHox-7*) is expressed in high amounts by the undifferentiated mesenchymal cells of the developing chick limb bud that are being maintained in an actively proliferating, labile undifferentiated condition

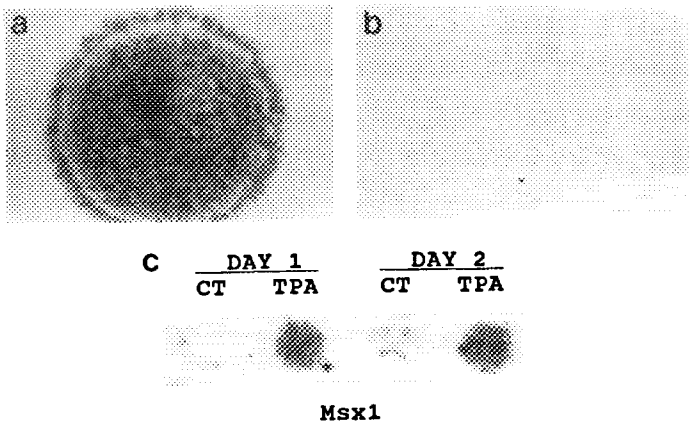

Fig. 1. (a,b) Accumulation of Alcian blue, pH 1.0-positive cartilage matrix by control (a) and TPA-treated (b) limb mesenchymal cell micromass cultures. TPA (0.1 mM) completely inhibits the accumulation of Alcian blue-stainable cartilage matrix. (c) Autoradiograph demonstrating the hybridization of a *Msx1*-specific cDNA to RNA sequences in the cytoplasm of day 1 and day 2 control (CT) and TPA-treated limb mesenchymal cell micromass cultures. Equivalent amounts of poly(A)+ RNA were applied in each well. Control cultures exhibit no detectable expression of *Msx1* mRNA, whereas *Msx1* mRNA is expressed in TPA-treated cultures.

by the apical ectodermal ridge at the distal periphery of the limb bud [see 11 for review]. In the developing chick limb bud, the expression of *Msx1* by undifferentiated subridge mesenchymal cells is regulated by the AER, and its expression ceases coincident with the onset of limb chondrogenic differentiation [11]. Thus, it has been suggested that *Msx1* may be involved in suppressing the differentiation of the subridge mesenchymal cells of the limb bud, and that the cessation of *Msx1* expression may be required for terminal differentiation to occur [11]. As shown in Fig. 1c, control limb mesenchymal cell micromass cultures, which uniformly undergo chondrogenesis, exhibit no detectable expression of *Msx1* mRNA. In contrast, *Msx1* mRNA expression is maintained in TPA-treated cultures which fail to undergo chondrogenesis (Fig. 1c).

Thus, cells treated with the tumor promoting phorbol ester, TPA exhibit deregulated expression of a Hox gene that has been implicated in suppressing the differentiation of limb mesenchymal cells. In this regard, it is noteworthy that forced expression of *Msx1* blocks the terminal differentiation of myogenic precursor cells, while conferring the cells with oncogenic potential such that they exhibit a transformed phenotype and form tumors when injected into nude mice [22]. These observations, as well as those described in the present study, suggest that the acquisition of tumorigenic potential by cells may involve the deregulated expression of regulatory genes such as Hox genes which are normally suppressed in differentiated cells and which endow cells with the lability characteristic of undifferentiated embryonic cells. In this regard, it is noteworthy that *Msx2* (formerly called *Hox-8*), a Hox gene closely related to *Msx1*,

is aberrantly expressed by epithelial tumors [23], and several other tumors are characterized by the deregulated expression of various Hox genes [24-26].

Since the tumor promoting activity of phorbol esters in a variety of cell types correlates with an impairment in gap junctional intercellular communication (see ref. 6 for review], we examined the effect of TPA on intercellular communication via gap junctions in limb mesenchymal cell micromass cultures. The onset of cartilage differentiation in the developing chick limb is characterized by a transient cellular condensation process during which intimate cell-cell interactions occur which are required to trigger chondrogenic differentiation [27,28]. Extensive intercellular communication via gap junctions occurs during condensation and the onset of the chondrogenic differentiation of limb mesenchymal cells in vitro, and it has been suggested that gap junctional communication may be involved in regulating the onset of limb cartilage differentiation [12].

We examined the effect of TPA on the intercellular transfer of lucifer yellow dye via gap junctions in limb mesenchymal cell micromass cultures by scrape-loading/dye transfer. As shown in Fig. 2a, extensive transfer of lucifer yellow dye from scrape-loaded cells to contiguous cells

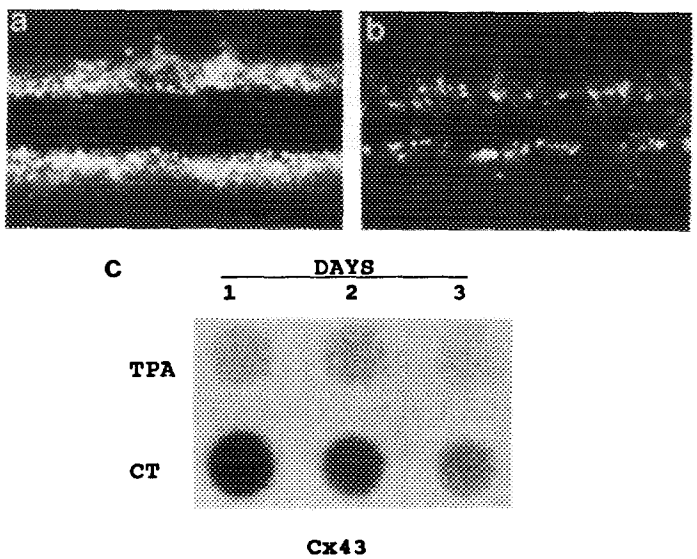

Fig. 2. (a,b) Fluorescence photomicrographs demonstrating the intercellular transfer of lucifer yellow dye via gap junctions from scrape-loaded cells to contiguous cells in control (a) and TPA-treated (b) limb mesenchymal cell micromass cultures. Extensive transfer of lucifer yellow dye from scrape-loaded cells to contiguous cells occurs in control cultures (a), whereas little or no transfer of lucifer yellow from scrape-loaded cells to contiguous cells occurs in TPA-treated micromass cultures (b). (c) Autoradiograph demonstrating the hybridization of a connexin43 (Cx43) -specific cDNA to RNA sequences in the cytoplasm of day 1, 2, and 3 control (CT) and TPA-treated limb mesenchymal cell micromass cultures. Equivalent amounts of poly(A)+ RNA were applied in each well. The relative abundance of Cx43 mRNA/total poly(A)+ RNA as determined by densitometry is about 4-fold greater in control cultures than in TPA-treated cultures on day 1; 2.5-fold greater on day 2; and, 1.5-fold greater on day 3.

occurs in control micromass cultures at the onset of their chondrogenic differentiation. In contrast, little or no transfer of lucifer yellow dye from scrape-loaded cells to contiguous cells occurs in TPA-treated micromass cultures (Fig. 2b). The inhibition of intercellular transfer of lucifer yellow via gap junctions in TPA-treated cultures is accompanied by a dramatic reduction in the steady-state levels of mRNA for the gap junction protein Cx43 (Fig. 2c).

Thus, TPA inhibits gap junctional communication and Cx43 expression by limb mesenchymal cells, while inhibiting their chondrogenic differentiation. These observations are consistent with several previous studies suggesting that the tumor promoting activity of phorbol esters may relate to their ability to disrupt the intercellular communication that may be required to initiate and/or maintain the differentiated phenotype of cells [3-6]. In this regard, it is noteworthy that most, if not all, cancer cells exhibit aberrant gap junctional communication among themselves or with surrounding normal cells [6], and activation of several oncogenes leads to suppression of gap junctional communication [5,29-31].

In summary, we have found that limb mesenchymal cells inhibited from undergoing chondrogenic differentiation by the tumor promoter, TPA exhibit deregulated expression of *Msx1*, a Hox gene that has been implicated in suppressing the differentiation of limb mesenchymal cells, and also fail to exhibit the extensive gap junctional intercellular communication that normally occurs at the onset of limb cartilage differentiation. These observations provide support for the hypotheses that the tumor promoting activity of phorbol esters may relate to their ability to modulate the expression of regulatory genes that are involved in controlling terminal differentiation, as well as to their ability to disrupt the intercellular communication that may be involved in initiating and/or maintaining the differentiated phenotype of cells. Since carcinogenesis is a complex multistage process [1,2] either of these mechanisms of action of tumor promoters may play a role in the carcinogenic process.

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REFERENCES

1. Weinstein, I. B. (1988) Cancer Research 48, 4135-4143.
2. Weinstein, I. B. (1991) Env. Health Persp. 93, 175-179.
3. Murray, A. W. and Fitzgerald D. J. (1979) Biochem. Biophys. Res. Commun. 91, 395-401.
4. Yotti, L. P., Chang, C. C. and Trosko, J. E. (1979) Science 20, 1089-1091.
5. Brissette J. L., Kumar, N. M., Gilula, N. B. and Dotto G. P. (1991) Mol. Cell. Biol. 11, 5364-5371.
6. Yamasaki, H. (1991) Env. Health Persp. 93, 191-197.
7. Biddulph D. M. and Dozier, M. M. (1989) Exp. Cell Res. 185, 541- 545.
8. Sasse, J., von der Mark, K., Pacifici, M., and Holtzer, H. (1983) Prog. Clin. Biol. Res. 110B, 159-166.
9. Tsonis, P. A. and Goetinck P. F. (1990) Exp. Cell Res. 190, 247-253.

10. Kulyk, W. M. (1991) Dev. Biol. 146, 38-48.
11. Coelho C. N. D., Sumoy, L., Rogina, B., Upholt, W. B. and Kosher, R. A. (1993) Prog. Clin. Biol. Res. 383A, 61-70.
12. Coelho, C. N. D. and Kosher, R. A. (1991) Dev. Biol. 144, 47-53.
13. Hamburger, V. and Hamilton, H. L. (1951) J. Morph. 88, 49-92.
14. Gay, S. W. and Kosher, R. A. (1984) J. Exp. Zool. 232, 317-326.
15. El-Fouly, M. H., Trosko, J. E. and Chang, C. C. (1987) Exp. Cell Res. 168, 422-430.
16. White, B. A. and Bancroft, F. C. (1982) J. Biol. Chem. 257, 8569-8572.
17. Kosher, R. A., Gay, S. W., Kamanitz, J. R., Kulyk, W. M., Rodgers, B. J., Sai, S., Tanaka, T. and Tanzer, M. L. (1986) Dev. Biol. 118, 112-117.
18. Leonard, C. M., Fuld, H. M., Frenz, D. A., Downie, S. A., Massague, J. and Newman, S. A. (1991) Dev. Biol. 145, 99-109.
19. Coelho, C. N. D., Sumoy, L., Kosher, R. A. and Upholt, W. B. (1992) Differentiation 49, 85-92.
20. Dealy, C. N., Beyer, E. C. and Kosher, R. A. (1994) Developmental Dynamics 199, 156-167.
21. Harley, C. B. (1987) Gene Anal. Tech. 4, 17-22.
22. Song, K., Wang, Y. and Sassoon, D. (1992) Nature 360, 477-481.
23. Suzuki, M., Tanaka, M., Iwase, T., Naito, Y., Sugimura, H. and Kino I. (1993) Biochem. Biophys. Res. Commun. 194, 187-193.
24. Aberdam, D., Negreanu, V., Sachs, L. and Blatt C. (1991) Mol. Cell. Biol. 11, 554-557.
25. Hatano, M., Robert, C. W. M., Minden, M., Crist, W. M. and Korsmeyer, S. J. (1991) Science 253, 79-82.
26. Cillo, C., Barba, P., Freschi, G., Bucciarelli, G., Magli, M. C. and Boncinelli, E. (1992) Int. J. Cancer 51, 892-897.
27. Kosher, R. A. (1983) In Cartilage (B. K. Hall, Ed.), Vol. 1, pp. 59-85, Academic Press, New York.
28. Solursh, M. (1983) In Cartilage (B. K. Hall, Ed.), Vol. 2, pp. 121-141, Academic Press, New York.
29. Azarnia, R. S., Reddy, S., Kmiecik, T. E., Shalloway, D. and Loewenstein, W. R. (1988) Science 239, 398-401.
30. Bignami, M., Rosa, S., Falcone, G., Tato, F., Katoh, F. and Yamasaki, H. (1988) Mol. Carcinogenesis 1, 67-75.
31. El-Fouly, M., Trosko, J. E., Chang, C. C. and Warren, S. T. (1989) Mol. Carcinogenesis 2, 131-135.