TWO-STAGE MALIGNANT TRANSFORMATION IN HAMSTER EMBRYO CELLS

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Summary.—Transformation of primary hamster embryo cells was investigated using 3-methylcholanthrene (MCA), a combination of MCA and 12-O-tetradecanoylphorbol-13-acetate (TPA), and initiation with MCA or dibenz(a,h)anthracene (DBA) followed by promotion with TPA. Evidence for transformation was (a) abnormal cellular morphology, (b) increased lifespan, (c) growth in soft agar, and (d) tumour induction by s.c. inoculation into suckling hamsters.

Cells treated with either MCA or MCA + TPA showed the same latent period to morphological transformation, although their tumorigenic potential varied. Cells did not form tumours when TPA was administered 7 days after treatment with either MCA or DBA. However, when administration of TPA was delayed to 27 days after treatment with a transforming dose of MCA or a subthreshold dose of DBA, the cells transformed and produced tumours in hamsters.

Our results show that TPA may act as an inhibitor or promoter, depending on the length of time between treatment of the hamster embryo cells with the carcinogen and administration of the TPA. It appears that treatment of cells with TPA before the initiating event is complete inhibits or delays the development of their ability to induce tumours in animals or grow in soft agar. However, with a sufficient interval between the application of the initiating carcinogen and the promoter, transformation occurs, and the ability of cells treated with subthreshold doses of DBA to form tumours is enhanced.

The ability of promoting agents to enhance the formation of tumours in mouse skin by carcinogens has been reviewed by Boutwell (1964, 1974). In vitro effects of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on cellular multiplication, RNA synthesis and DNA repair synthesis have been studied in mouse fibroblasts (Sivak & Van Duuren, 1970; Sivak et al., 1969, 1972; Trosko et al., 1975). More recently, TPA has been shown to enhance the focal transformation rate of mouse C3H/10T1/2 cells (Mondal & Heidelberg, 1976; Mondal et al., 1976) and to enhance transformation in rat fibroblasts in culture (Lasne et al., 1974, 1977). The rate of focal transformation in mouse cells could be increased or decreased, depending on the time at which TPA was administered relative to the carcinogen (Mondal et al., 1976). Our studies were undertaken to determine whether application of the promoting agent, TPA, would reduce the time from treatment with a carcinogen to the appearance of neoplastic transformation of hamster embryo cells in vitro.

Since TPA can alter the morphology of human, mouse and chick cells (Sivak, et al., 1969; Diamond et al., 1974) we studied the effects of TPA on the transformation of hamster cells, as measured by the presence of foci of piled up cells in the monolayer cultures. Subsequently, as a measure of their malignancy, we investigated the ability of these cells to grow in soft agar and induce tumours in animals.

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MATERIALS AND METHODS

Cells.—Primary cell pools 1839, 583 and 241 were prepared from random-bred Syrian golden hamster embryos (ELA/ENG) (Engle Laboratory Animals, Farmersburg, IN) at 13 days of gestation. The cells were planted at 2 × 10^7/150 cm² flask (Costar Products, Cambridge, MA) in Dulbecco’s Modified Eagle’s Medium (DMEM) (Grand Island Biological Corp., Grand Island, NY) supplemented with 2 mM L-glutamine and 10% heat-inactivated foetal calf serum (Reheis Chemical Co., Phoenix, AZ). Only serum determined by the Viral Resources Laboratory (FCRC, Frederick, MD) to be free from mycoplasma and bacteriophage was used. All cell cultures were fed 3 times per week. Cells were disaggregated with ENZAR T (Reheis) when 90% confluent.

Chemicals.—Stock solutions of 3-methylcholanthrene (MCA) and dibenz(a,h)anthracene (DBA) (Eastman Kodak, Rochester, NY) and TPA (Consolidated Midland Corp., Brewster, NY) were prepared in dimethylsulphoxide (DMSO) (Crown Zellerbach Corp., Camas, WA). The chemicals were further diluted in DMEM to obtain the required dose in a final concentration of 0-2% DMSO.

Simultaneous treatment with MCA and TPA.—Cells from culture 1839 were seeded at 5 × 10⁶/75 cm² flask 4 h before chemical treatment with either DMEM, DMEM + 0-2% DMSO, DMEM + 0-5 μg/ml MCA, DMEM + 0-1 μg/ml TPA or the combination TPA + MCA. Cultures were maintained at 37°C in a humidified incubator in an atmosphere of 10% CO₂ in air. Subcultures were made by dividing the cells 1:2 and 1:4. Cultures reaching confluence after a 1:2 subdivision were considered to have undergone one population doubling (PD), 2PD after a 1:4 subdivision, and 4 PD after a 1:16 subdivision. Cells were exposed to both carcinogen and TPA continuously for 30 days. Thereafter, all cultures were maintained in DMEM alone.

Simultaneous treatment with MCA and varying doses of TPA.—Following the same procedure as described above, cell culture 583 was treated with DMEM, DMEM + 0-2% DMSO, 0-01, 0-1, or 1 μg/ml of TPA alone or in combination with MCA. Chemical treatment was continued for 30 days, followed by maintenance on DMEM alone.

Carcinogen treatment followed by TPA treatment.—Cells from culture 241 were seeded at 5 × 10⁶/75 cm² flask and after 4 h treated for 48 h as follows: (1) DMEM, (2) DMEM + 0-2% DMSO, (3) 0-5 μg/ml MCA and (4) 1-0 μg/ml DBA. On Day 2 the cultures were re-fed with DMEM and allowed to grow to confluence (Day 6). They were then subcultured into 2 groups. Twenty-four hours after subculture (Day 7) one flask from each group was treated with 0-1 μg/ml TPA and the other re-fed with DMEM, producing 8 groups. At subculture, 2 flasks were made for each treatment regime. No toxicity was seen in any of the cultures, and all cultures reached confluence within 48 h of a 1:2 subdivision. Therefore, split ratios were increased to a level which allowed all cultures to become confluent within 7 days. The population doubling level was adjusted according to the subculture ratio 1:16. On Day 30, half of each group of flasks which had been treated with TPA were re-fed with DMEM and remained on DMEM for the rest of the experiment. At the same time (Day 30), half of each group of flasks which had received only the 48 h chemical were treated with TPA. Treatment was continued throughout the rest of the experiment. The remaining flasks in this group were never exposed to TPA. This produced 16 groups.

Transformation.—Morphological transformation was defined as an increase in cell density accompanied by numerous cells growing suspended in the medium as well as on the surface of the cell sheet forming foci, with a loss of polar orientation of the cells on the monolayer. After morphological transformation was seen, 10⁶ cells from each culture were seeded in soft agar (MacPherson, 1969) and 10⁶ cells were injected s.c. into 9-day-old suckling hamsters. Soft agar cultures were held for 45 days unless macroscopic colonies were seen earlier. Inoculated hamsters were held for 90 days before being assessed as negative for tumour formation.

RESULTS

Effects of simultaneous treatment with MCA and TPA

Cells from culture 1839 which were treated with MCA or MCA + 0-1 μg/ml TPA showed morphological transformation by Day 30. At PD 20, the cells were plated into soft agar and injected into suckling hamsters. There was an increase
in the latent period to tumour formation, and a slightly lower incidence of tumour induction, in hamsters receiving (MCA + 0-1 µg/ml TPA)-treated cells compared with those receiving cells treated with MCA alone (Table I). No tumours were seen in hamsters receiving cells from cultures treated with DMEM, DMSO, or 0-1 µg/ml TPA alone. Only cells from those cultures treated with MCA alone produced rapidly growing colonies in soft agar.

**Effects of simultaneous treatment with MCA and varying doses of TPA**

Culture 583 treated with MCA or MCA + TPA (at any of the doses used) showed morphological transformation, whilst control cultures, or those receiving TPA alone, did not (Table II). (This cell culture was characterized by a longer latent period to morphological transformation than culture 1839). At PD 20, the cells were plated in soft agar and injected into suckling hamsters. Culture 583 responded similarly to culture 1839 when treated with MCA or MCA + 0-1 µg/ml TPA. When it was treated with MCA + 0-01 or 1 µg/ml TPA; or with TPA alone, the cells failed to induce tumours in animals or grow in soft agar. This was also true of untreated controls. All cultures which had not reached senescence at PD 40 were retested for growth in soft agar and tumorigenicity. Cultures treated with MCA, MCA + 0-01 or 0-1 µg/ml TPA grew in soft agar and produced tumours in hamsters. The only remaining non-tumourigenic culture which had not reached senescence (MCA + 1 µg/ml TPA) grew in soft agar (<0-1%) and produced tumours in animals when it was retested at PD 64.

**Effects of carcinogen treatment followed by TPA treatment**

Of the 16 different treatment regimens, only 4 produced malignantly transformed cells which grew in soft agar and produced tumours in animals. These were from cells treated with (1) MCA for 48 h, (2) MCA for 48 h followed by TPA at PD 30, (3) DBA for 48 h followed by TPA at Days 7-30 and (4) DBA for 48 h followed by TPA at Day 30 (Table III). As in the 2 studies mentioned above, no transformation occurred when cells were treated with DMEM, DMEM + 0-2% DMSO or DMEM + 0-1 µg/ml TPA. All the cultures treated with TPA at different times after treatment with DMEM or DMEM + 0-2% DMSO responded similarly, and one representative group is included in the table. Cells which transformed when treated with MCA followed by TPA at Day 30 had a slightly shorter latency than those transformed by MCA alone. Similarly, cultures which transformed after treatment with DBA followed on Day 30 by TPA had a shorter latency than those transformed after treatment with DBA.

### Table I. — Effects of simultaneous treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 3-methylcholanthrene (MCA) on transformation of hamster embryo cells

| Treatment (2 flasks) | Morphological transformation | Growth in soft agar (%) | Terminal PD | Tumorigenicity* (PD20 latency) |
|----------------------|-----------------------------|-------------------------|-------------|-------------------------------|
| DMEM†                | None                        | —‡ ND                   | 20          | 0/4                           |
| DMSO                 | None                        | — ND                    | 20          | 0/6                           |
| TPA (0-1 µg/ml)      | None§                       | — ND                    | 34          | 0/9                           |
| MCA (0-5 µg/ml)      | (PD9) 30 days               | 3 (7 days)              | >95         | 6/6 (7 days)                  |
| MCA + TPA            | (PD9) 30 days               | 2 (7 days)              | >95         | 5/7 (20 days)                 |

* Number of hamsters with tumours/number inoculated; all tumours anaplastic spindle-cell sarcomas.
† Abbreviations used: DMEM = Medium control; DMSO = Solvent control; ND = Not determined (cultures had terminated); PD = Population Doubling.
‡ Negatives maintained in culture for 45 days.
§ Retained the rapid growth rate, orientation and characteristics of earlier-passage control cells.

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Table II.—Effects of simultaneous treatment with MCA and various doses of TPA on transformation of hamster embryo cells.

| Treatment                                  | Morphological transformation | % Growth in soft agar | Terminal PD | Tumorigenicity* (latency) |
|--------------------------------------------|------------------------------|-----------------------|-------------|--------------------------|
| DMEM†                                      | None                         | —                     | 23          | 0/5                     |
| DMSO                                       | None                         | —                     | 18          | 0/8                     |
| TPA (0.01 µg/ml)                           | None                         | —                     | 34          | 0/7                     |
| TPA (0.1 µg/ml)                            | None                         | —                     | 31          | 0/8                     |
| TPA (1.0 µg/ml)                            | None                         | —                     | 16          | ND                      |
| MCA (0.5 µg/ml)                            | (PD19) 56 days               | 3 (7 days)            | >95         | 9/9 (7 days)            |
| MCA + TPA (0.01 µg/ml)                     | (PD19) 56 days               | —                     | >95         | 0/20                    |
| MCA + TPA (0.1 µg/ml)                      | (PD19) 56 days               | —                     | >95         | 8/21                    |
| MCA + TPA (1.0 µg/ml)                      | (PD19) 56 days               | —                     | >95         | 0/11                    |

* Number of hamsters with tumours/number inoculated; all tumours anaplastic spindle-cell sarcomas.
† Abbreviations used: as in Table I.
‡ Negatives maintained in culture for 45 days.
### Table III.—Effects of TPA on hamster embryo-cell transformation by MCA and dibenz(a,h)anthracene (DBA)

| Treatment (2 flasks) | TPA (0.1 µg/ml) | Morphological transformation | Growth in soft agar | Tumorigenicity* (latency) |
|----------------------|-----------------|----------------------------|---------------------|--------------------------|
|                      |                 |                            | %       | PD | PD | PD |  |
| DMEM                 | None            | None                       | —        | 35 | 44 | 35 | 0/6 |
| DMSO                 | None            | None                       | —        | 35 | 42 | 35 | 0/9 |
| DMSO‡                | PD2 continuously from Day 7 | None                       | —        | 60 | 70 | 60 | 0/9 |
| MCA (0.5 µg/ml)      | None            | (PD29) 56 days             | 3        | 53 | >95 | 54 | 5/13 (7 days) |
| MCA                  | PD2 continuously from Day 7 | None                       | —        | 57 | >95 | 60 | 0/18 |
| MCA                  | PD2–PD13 from Days 7 to 30 | None                       | —        | 57 | >95 | 60 | 0/18 |
| MCA                  | PD13 continuously from Day 30 | (PD21) 45 days             | 3        | 57 | >95 | 56 | 10/10 (25* days) |
| DBA (1.0 µg/ml)      | None            | None                       | —        | 58 | 67 | 59 | 0/10 |
| DBA                  | PD2 continuously from Day 7 | None                       | —        | 57 | >95 | 56 | 0/12 |
| DBA                  | PD2–PD13 from Days 7 to 30 | (PD33) 65 days             | 0.01     | 59 | >95 | 60 | 11/11 (25 days) |
| DBA                  | PD13 continuously from Day 30 | (PD21) 45 days             | 2        | 53 | >95 | 56 | 3/7 (18* days) |

* Number of hamsters with tumours/number inoculated; all tumours anaplastic spindle-cell sarcomas.
† Negatives maintained for 45 days.
‡ Representative of 6 control groups receiving the same sequence of TPA as those groups treated with carcinogen followed by TPA.
followed by TPA on Days 7–30. Neither of the cultures treated with MCA or DBA and followed by continuous treatment with TPA from Day 7 grew in soft agar or produced tumours in hamsters, although a terminal PD was not reached (>95 PD’s).

**DISCUSSION**

Using hamster embryo cells, we studied the effect of TPA treatment on malignant transformation. Parameters measured included: morphological transformation, increased lifespan, growth in soft agar and induction of tumours in animals. Our studies show that TPA can act either as an inhibitor or a promoter of malignant transformation in hamster embryo cells depending on the time interval between treatment with carcinogen and the administration of TPA. Similarly, TPA-dependent stimulation or inhibition of the rate of focal transformation of mouse fibroblasts (C3H/10T 1/2) cells was shown to depend upon the length of time between carcinogen and TPA treatments (Mondal et al., 1976). When the hamster cells were treated with MCA or MCA + TPA, morphological transformation was observed at the same time irrespective of treatment. Similarly, all the groups showing morphological transformation grew beyond the lifespan of the control cultures. However, induction of tumours in animals did not occur until 20 PD later with the lowest TPA concentration, and 44 PD later with the highest TPA concentration. Similar observations were made concerning growth in soft agar. When TPA was administered simultaneously with MCA, criteria indicative of malignant transformation were inhibited.

Treatment with TPA 7 days after MCA produced complete inhibition of both tumour formation and growth in soft agar. In contrast to our observations, others have shown that when TPA was applied 3–5 days after carcinogen treatment in mouse cells (Mondal et al., 1976) or 7 days after carcinogen treatment in rat cells (Lasne et al., 1977) enhanced rates of focal transformation occurred.

Treatment with TPA 27 days after MCA treatment reduced the latent period to morphological transformation and increased the tumour incidence. Perhaps a longer time is required to complete the first stage of transformation in hamster than in mouse (Mondal et al., 1976) or rat cells (Lasne et al., 1977). Treatment with TPA simultaneously with, or soon after, treatment with a transforming dose of MCA may prevent the repair of carcinogen damage to the cells.

A promoting effect was found when hamster embryo cells were treated with a subtransforming dose of DBA and TPA was administered either between 7 and 27 days, or from 27 days after DBA treatment. However, in all experiments with a transforming dose of carcinogen, the continued presence of TPA in the culture medium during the first 30 days appeared to have inhibited the neoplastic transformation of the cells. Cells acquired the properties of growth in soft agar and tumour production in animals only after TPA had been removed, or when treatment with TPA did not begin until 27 days after treatment with a transforming dose of MCA.

Morphological changes appeared to reflect the initial events in transformation that are followed later by tumorigenesis and growth in soft agar. The studies reported here indicate that cells become tumorigenic before they acquire the ability to grow in soft agar (Tables I and II). This confirms the use of the generally observed in vitro characteristic of growth in soft agar as a valid indicator of the tumorigenic potential of cells. The inhibitory effects of TPA on characteristics of transformation may also occur through an inhibition of the mechanisms responsible for invasive properties of the transformed cells. The use of TPA in combination with MCA or DBA on hamster embryo cells to delay the appearance of malignant characteristics provides a model system for studying the sequence of events from morphological transformation to tumourigenicity.
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REFERENCES

BOUTWELL, R. K. (1964) Some biological aspects of skin carcinogenesis. Prog. Exp. Tumor Res., 4, 207.
BOUTWELL, R. K. (1974) The function and mechanism of promoters of carcinogenesis. CRC Crit. Rev. Toxicol., 2, 419.

DIAMOND, L., O'BRIEN, S., DONALDSON, C. & SHIMIZU, Y. (1974) Growth stimulation of human diploid fibroblasts by the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate. Int. J. Cancer, 13, 721.
LASEN, C., GENTIL, A. & CHOUROUINKOV, I. (1974) Two-stage malignant transformation of rat fibroblasts in tissue culture. Nature, 247, 490.
LASEN, C., GENTIL, A. & CHOUROUINKOV, I. (1977) Two-stage carcinogenesis with rat embryo cells in tissue culture. Br. J. Cancer, 35, 722.
MACPHERSON, I. (1969) Agar suspension culture for quantitation of transformed cells. In Fundamental Techniques in Virology. Eds K. Abel and N. P. Salzman. New York: Academic Press, p. 214.
MONDAL, S. & HEIDELBERGER, C. (1976) Transformation of C3H/10T1/2C18 mouse embryo-fibroblasts by ultraviolet irradiation and a phorbol ester. Nature, 260, 710.
MONDAL, S., BRANKOW, D. W. & HEIDELBERGER, C. (1976) Two-stage chemical oncogenesis in cultures of C3H/10T1/2 Cells. Cancer Res., 36, 2254.
SIVAK, A. & VAN DUUREN, B. L. (1970) RNA synthesis induction in cell culture by a tumor promoter. Cancer Res., 30, 1203.

SIVAK, A., MASSMAN, B. T. & VAN DUUREN, B. L. (1972) Activation of cell membrane enzymes in the stimulation of cell division. Biochem. Biophys. Res. Commun., 46, 606.
SIVAK, A., RAY, F. & VAN DUUREN, B. L. (1969) Phorbol ester tumor-promoting agents and membrane stability. Cancer Res., 29, 624.
TROSKO, J. E., YAGER, J. D. JR., BOWDEN, J. T. & BUTCHER, F. R. (1975) The effects of several croton oil constituents on two types of DNA repair and cyclic nucleotide levels in mammalian cells in vitro. Chem-Biol. Interact., 11, 191.