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Effects of 12-O-tetradecanoylphorbol-13-acetate on the incorporation of labelled precursors into RNA, DNA and protein in epidermis, dermis and subcutis from precancerous mouse skin with reference to enhanced tumorigenesis

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The effects of a single application of 1.8 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA) on precursor incorporation into RNA, DNA and protein in the epidermis, dermis and subcutis from 3-methylcholanthrene (MCA) injected precancerous mouse skin were studied at various time points between 3 and 96 h. In the precancerous tissues, the rates of incorporation of [^{3}H]uridine into RNA did not alter appreciably from those in the control tissues; while the rates of [^{3}H]methylthymidine incorporation into DNA were elevated with peaks appearing between 6 and 12 h, at 24 h and at 72 h in epidermis, dermis and subcutis. The rate of incorporation of [^{14}C]leucine into protein was markedly elevated in all the three tissues which showed 3 – 4 sharp peaks. The maximum stimulation ranged between 14 and 20 times that of the control. A single application of TPA to the precancerous mouse skin induced early stimulation of precursor incorporation into all the three macromolecules in epidermis, dermis and subcutis. The increased stimulation was maintained for 36 – 72 h. The patterns of incorporation of [^{3}H]methylthymidine into DNA gave rise to 2 – 3 peaks of elevated uptake in each tissue up to 36 – 48 h. A lowered rate of DNA synthesis between 48 and 60 h was followed by a peak at 72 h. In each group, epidermal mitotic activity correlated well with spurts of precursor incorporation into cellular DNA. The observations indicate that TPA recruits more cells into the DNA synthetic phase and accelerates selective growth of preneoplastic cells during tumor progression.

Introduction

It is known that the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)* induces waves of epidermal cell proliferation and differentiation when applied to mouse skin (1 – 3). The kinetics of nucleic acids and protein synthesis in the interfollicular epidermal cells and whole mouse skin treated with a strong tumor promoting dose of TPA, investigated by various workers, indicate that a single application of TPA to normal mouse skin causes sequential stimulation of RNA, protein and DNA synthesis similarly to that observed in actively growing tissues (2,4,5). However, the response of precancerous mouse skin to TPA with respect to macromolecular synthesis has not been reported. An earlier study from this laboratory showed that one subcutaneous injection of 0.5 mg 3-methylcholanthrene (MCA) to six-week-old female Swiss albino mice induces fibrosarcomas, carcinomas and rhabdomyosarcomas in a decreasing order of frequency in the back skin (6). In an extension of this study, histology of the tumors induced in mice receiving twice weekly applications of 1.8 nmol TPA in 0.1 ml acetone or 0.1 ml acetone, commencing six weeks after carcinogen delivery was compared with that of tumors obtained in mice treated with MCA only (7,8). A small dose of 1.8 nmol TPA was used as the skin was exposed to a completely carcinogenic dose of MCA. TPA treatment was started six weeks after MCA injection, the period usually required for the occurrence of precancerous change or an occasional palpable neoplasm (6). The tumor induction data showed that twice weekly application of TPA to MCA injected mouse skin enhances the incidence of epithelial as well as mesenchymal tumors such as carcinoma, trichoepithelioma, rhabdomyosarcoma, hibernoma and liposarcoma (7,8).

A preliminary investigation on the effects of a single application of TPA on the morphology, nucleic acids and protein levels in epidermis, dermis and subcutis from untreated and MCA injected skin showed significantly decreased levels of DNA in the epidermis and subcutis of MCA injected mice as compared to the same tissue site from untreated controls. TPA application however resulted in epidermal hyperplasia, marked inflammatory changes and significantly increased levels of DNA in all the three skin components from normal as well as carcinogen injected mice (9). These observations and the fact that the histologically diverse tumors originate from cells in different layers of skin have prompted the investigation repprted here, on the incorporation of precursors into nucleic acids and protein in the separated constituents of precancerous mouse skin. Effects of TPA on these parameters have been studied in an attempt to understand its mode of action in enhancing skin tumorigenesis.

*Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate: MCA, 3-methylcholanthrene; TFB, thiophene-free benzene; TCA, trichloroacetic acid; PCA, perchloric acid.

Materials and methods

Chemicals

MCA (Koch-Light and Co. Ltd., UK) was dissolved in thiophene free benzene (TFB) to a concentration of 0.25%. TPA was obtained as a gift from Dr. B.L. Van Duuren of New York University Medical Center, NY. It was used at a concentration of 1.8 nmol in 0.1 ml acetone. [^{3}H]Uridine-5-T (sp. act. 10 900 mCi/mmol), [^{3}H]methylthymidine (sp. act. 10 600 mCi/mmol) and [^{14}C]L-leucine (sp. act. 53.7 mCi/mmol) were obtained from the Isotope division of the Bhabha Atomic Research Centre, Bombay, India.

Animals

Six- to seven-week-old female Swiss albino mice were used in all the experiments. This strain was obtained originally from the Rockefeller Institute, NY and has completed several generations of controlled inbreeding at the Cancer Research Institute, Bombay. The animals, housed in air conditioned rooms with a constant day-night rhythm received adequate protein diet and water *ad libitum*.

The animals were given a s.c. injection of 0.5 mg MCA in 0.2 ml thiophene free benzene in the right scapular region. This dose effectively induces both epithelial and mesenchymal skin tumors and is non-toxic (6). Control animals were similarly treated with TFB. Six weeks after MCA injection 1.8 nmol TPA in 0.1 ml acetone or 0.1 ml acetone was applied to the reactive skin at the site of MCA injection. Hair on the back skin was shaved with an electric clipper 48 h prior to treatment, wounded animals being excluded from the experiment. 75 µCi [^{3}H]uridine, 33 µCi [^{3}H]methylthymidine or 5 µCi [^{14}C]L-leucine in 0.1 ml of balanced salt solution was injected i.p. 1 h prior to sacrificing the aimals (4). Remnant hair was removed by application of a depilatory cream for 3 min and washing thoroughly with water just before

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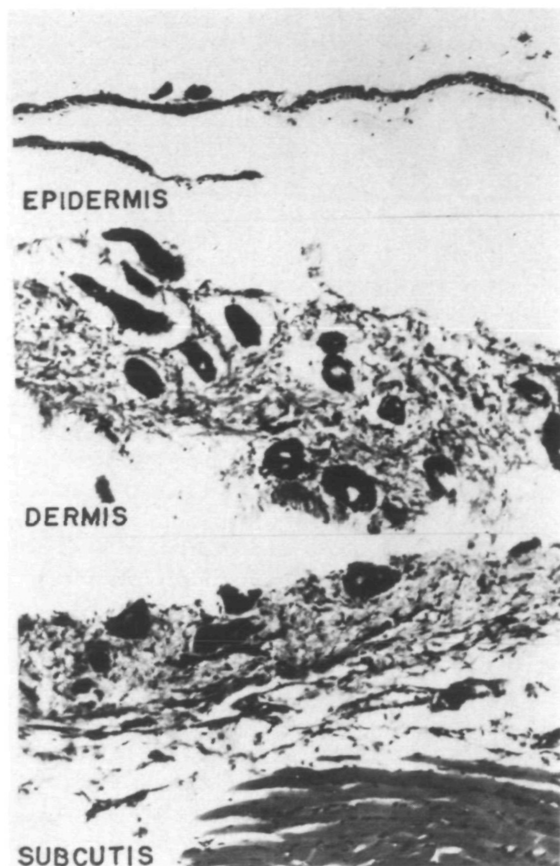

Fig. 1. Separated epidermis, dermis and subcutis from mouse skin. H & E, x 105.

killing the animals. Animals were sacrificed by cervical dislocation at various time points ranging between 3–96 h after TPA or acetone application. Separation of the epidermis, dermis and subcutis from the reactive area (~1–2 cm^2) was carried out (9) using a Watson's skin grafting knife with a suitably adjusted cutting angle (Figure 1). At each time point 4 groups of 3–4 animals each, treated either with TFB, MCA, MCA + TPA or MCA + acetone were sacrificed. The experiments were carried out in duplicate. Tissues from each group of animals were pooled in order to obtain sufficient material to carry out an experiment.

Extraction of DNA, RNA and protein from mouse epidermis, dermis and subcutis

For extraction of DNA and protein, tissues from mice injected with [^{3}H]-methylthymidine and [^{14}C]L-leucine were homogenized in ice cold distilled water and processed according to the method of Ogur and Rosen (10). After addition of 10% cold trichloroacetic acid (TCA), the homogenate was centrifuged at 5000 r.p.m. for 10 min at 4°C. The precipitate was then washed repeatedly with cold 5% TCA to remove the acid soluble fraction, 10% perchloric acid (PCA) was added to the precipitate and incubated at 0–4°C for 18 h after centrifugation. The precipitate was again centrifuged, washed with 5% PCA to remove lipids. Warm 10% PCA was added to the residue which was then heated at 90°C in a water bath for 30 min. The filtered supernatant was used for estimation of DNA by measurement of absorbance at 260 nm in a u.v. spectrophotometer (Beckmann) with calf thymus DNA as standard and for measurement of incorporation of [^{3}H]methylthymidine into DNA. The precipitate was further extracted with ether and the dried residue was solubilized in 20% ammonia and allowed to react for 4 h and filtered. The filtrate was used for estimation of protein and incorporation of [^{14}C]L-leucine. Protein was estimated by the method of Lowry *et al.* (11) using bovine serum albumin as standard.

Extraction of RNA

Tissues from mice injected with [^{3}H]uridine were homogenized in ice cold distilled water for preparing RNA by a modified Schmidt Thannhauser procedure (12). 0.6 N cold PCA was added to the homogenate which was then incubated at 0°C for 10 min and centrifuged at 10 000 r.p.m. for 10 min at 4°C. The pellet was washed twice with cold 0.2 N PCA, suspended in 4 ml 0.3 N KOH and incubated at 37°C for 18 h. The suspension was cooled, and allowed to stand at 0°C for 10 min after addition of 1.2 N PCA. The supernatant obtained after centrifugation contained RNA. The residue was washed twice with 0.2 N PCA and the washings added to the RNA fraction. RNA was estimated by measurement of u.v. absorbance at 260 nm using yeast RNA as standard.

Measurement of radioactivity

0.2 ml aliquots of each sample were counted in triplicate in 10 ml of Bray's fluid using Beckmann LS 100 Liquid Scintillation Counter. After making necessary correction for counting efficiency, the specific activity was expressed as d.p.m./μg nucleic acid or protein. Average specific activities of the macromolecules at each time point in TFB, MCA, MCA + acetone or MCA + TPA treated epidermis, dermis and subcutis are listed in Tables I, II and III. The average specific activity of nucleic acids and protein from the epidermis of animals receiving a single injection of MCA (precancerous skin) are expressed as percentage of average specific activity in TFB treated control in Figure 2A. Figure 2B shows the average specific activity of the macromolecules from MCA + acetone treated animals expressed as percentage of MCA treated control, while Figure 2C shows the average specific activity of nucleic acids and protein from MCA + TPA treated mouse epidermis expressed as percentage of MCA + acetone treated control. Data obtained on the dermis and subcutis are presented similarly in Figure 3 and Figure 4.

Histology

At each time point, 2 animals in each group receiving TFB, MCA, MCA + acetone or MCA + TPA were sacrificed. Reactive skin was fixed in 10% neutral formalin and 5 μ thick paraffin sections stained with haemotoxylin and eosin. Mitotic basal cells in the epidermis were counted at x 1000 and the mitotic index expressed on a count from 600–700 interfollicular basal cells, as the number of mitosis per 100 basal cells. The mitotic index data are listed in Table IV and expressed as percentage of control in Figure 5.

Results

Epidermis

In precancerous epidermis the rate of incorporation of [^{3}H]-uridine into RNA does not alter appreciably from that in solvent control animals. A slight depression between 3 and 24 h is followed by a single peak at 30 h (250% of the control), the level returning to control value at 36 h (Figure 2A). A single application of acetone to the precancerous skin causes mild stimulation of precursor incorporation into RNA at 3 h (138% of the control), followed by decreased levels up to 36 h with definite inhibition at 30 h (Figure 2B). Initially, TPA application causes a slight decrease in the rate of incorporation of [^{3}H]uridine into RNA at 3 h followed by maximum stimulation at 6 h (160% of the control), the level remaining at a low plateau above the control up to 18 h and reverting to control values between 24 and 48 h (Figure 2C).

Figure 2A shows that the rate of incorporation of [^{3}H]-methylthymidine into DNA is elevated in the precancerous epidermis. The first peak appears around 6–12 h (310–265% of the control), the second at 24 h (272% of the control) after which the levels return to control value and increase again to give rise to the third peak at 72 h (320% of the control). In the acetone treated epidermis the rate of precursor incorporation into DNA is slightly elevated at 12 h, 24 h and between 48 and 60 h and shows inhibition at 6 h and 72 h, returning to control level at 96 h (Figure 2B). As shown in Figure 2C, a single application of TPA produces a rapid and sustained stimulation of precursor incorporation into DNA between 3 and 72 h, decreasing to control level at 96 h. After early stimulation at 3 h, the first peak appears at 6 h (240% of the control) followed by a high plateau between 18 and 24 h (210% of the control) and a second peak at 36 h (188% of the control). The rate of precursor incorporation declines steadily to reach near control level at 48 h and increases again giving rise to the maximum peak at 72 h (246% of the control).

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Table I. Average specific activities of RNA, DNA and protein in the epidermis from mouse skin treated with TFB, MCA, MCA + acetone or MCA + TPA

Time in hours	Sp. act. d.p.m./μg RNA				Sp. act. d.p.m./μg DNA				Sp. act. d.p.m./μg protein			
	TFB only	MCA only	MCA + acetone	MCA + TPA	TFB only	MCA only	MCA + acetone	MCA + TPA	TFB only	MCA only	MCA + acetone	MCA + TPA
3	39.02	24.90	34.28	27.44	13.04	11.60	12.64	16.30	–	–	–	–
6	29.10	25.93	23.07	36.86	6.08	18.85	10.15	24.22	0.30	1.59	1.82	2.42
12	50.36	22.96	20.84	31.24	5.55	14.73	21.00	38.76	0.15	1.63	1.77	3.28
18	30.58	26.05	22.80	31.74	16.26	10.84	11.58	24.43	0.22	1.27	1.65	1.51
24	48.26	23.37	21.15	23.36	4.95	13.46	16.64	34.74	0.13	1.40	1.37	3.73
30	28.56	71.58	42.09	45.36	14.58	14.57	14.05	22.89	0.272	1.60	1.39	2.30
36	21.01	21.13	18.07	21.91	14.58	17.72	16.99	31.91	0.07	1.28	1.94	4.66
48	26.86	16.84	21.27	21.73	12.04	13.70	20.20	24.98	0.31	1.27	1.27	3.63
60	–	–	–	–	9.83	8.32	11.30	15.60	–	–	–	–
72	–	–	–	–	7.06	22.63	10.64	26.21	0.17	1.66	1.48	2.71
96	–	–	–	–	16.39	15.00	15.44	16.97	0.215	1.77	1.42	1.94

Table II. Average specific activities of RNA, DNA and protein in the dermis from mouse skin treated with TFB, MCA, MCA + acetone or MCA + TPA

Time in hours	Sp. act. d.p.m./μg RNA				Sp. act. d.p.m./μg DNA				Sp. act. d.p.m./μg protein			
	TFB only	MCA only	MCA + acetone	MCA + TPA	TFB only	MCA only	MCA + acetone	MCA + TPA	TFB only	MCA only	MCA + acetone	MCA + TPA
3	37.33	26.38	29.92	34.99	16.40	10.56	11.19	13.72	–	–	–	–
6	30.82	25.66	28.42	40.82	4.55	15.92	11.82	19.93	0.26	1.02	1.42	2.63
12	39.23	26.02	20.95	30.30	7.89	13.22	19.50	24.49	0.135	1.37	1.44	3.49
18	29.33	26.65	27.64	31.41	16.43	8.78	12.88	16.91	0.22	1.48	1.42	2.40
24	40.57	21.79	18.22	27.93	5.47	17.98	11.41	24.78	0.123	2.31	1.49	7.48
30	25.46	45.94	30.40	28.89	17.36	12.78	11.35	26.64	0.262	1.47	1.52	1.96
36	30.50	18.64	16.66	26.27	12.99	18.33	15.32	21.72	0.135	1.56	1.38	2.89
48	26.95	16.53	19.44	20.86	16.83	10.01	18.62	24.43	0.288	1.20	1.57	2.99
60	–	–	–	–	16.23	7.23	15.65	18.15	–	–	–	–
72	–	–	–	–	8.61	17.39	11.68	43.75	0.22	1.34	1.45	3.99
96	–	–	–	–	12.50	18.06	13.74	20.66	0.225	1.31	2.17	2.65

Table III. Average specific activities of RNA, DNA and protein in the subcutis from mouse skin treated with TFB, MCA, MCA + acetone or MCA + TPA

Time in hours	Sp. act. d.p.m./μg RNA				Sp. act. d.p.m./μg DNA				Sp. act. d.p.m./μg protein			
	TFB only	MCA only	MCA + acetone	MCA + TPA	TFB only	MCA only	MCA + acetone	MCA + TPA	TFB only	MCA only	MCA + acetone	MCA + TPA
3	48.07	18.55	28.45	33.12	6.70	14.28	10.62	8.32	–	–	–	–
6	36.67	25.00	27.61	31.32	2.50	17.97	11.71	24.12	0.125	1.27	1.45	1.73
12	55.36	26.07	28.88	26.43	8.33	21.53	25.21	21.85	0.065	1.33	1.63	2.64
18	38.89	28.35	24.36	53.85	16.26	11.30	10.50	13.41	0.155	0.94	1.42	1.15
24	45.02	24.56	25.32	33.93	6.33	18.58	20.08	15.49	0.15	2.29	2.13	3.91
30	29.99	48.14	38.48	41.22	22.20	12.11	14.15	23.71	0.239	1.23	1.83	1.61
36	31.28	26.52	22.34	24.79	16.64	21.52	15.91	25.36	0.145	2.09	2.13	2.45
48	35.75	24.76	21.96	23.65	20.75	13.80	25.36	20.83	0.375	1.19	2.13	2.02
60	–	–	–	–	30.92	26.79	15.07	18.46	–	–	–	–
72	–	–	–	–	8.22	12.82	14.05	28.88	0.136	0.86	1.63	2.73
96	–	–	–	–	15.86	25.62	20.51	18.06	0.295	2.00	1.36	2.05

Figure 2A shows that the rate of incorporation of [^{14}C]L-leucine into protein is elevated significantly in the precancerous epidermis. Four sharp peaks appear at 12 h, 24 h, 36 h and 72 h (1087%, 1077%, 1828% and 976% of the control, respectively), the level remaining well above control at 96 h (823% of the control). Acetone application slightly in-

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Table IV. Epidermal mitotic index

Time in hours	Number of mitosis per 100 basal cells			
	TFB only	MCA only	MCA + acetone	MCA + TPA
3	1.49	6.0	3.89	4.86
6	1.33	3.29	3.99	5.28
12	1.80	3.51	4.42	4.31
18	1.74	4.01	4.27	5.68
24	1.18	5.44	3.19	5.58
30	1.21	3.66	4.50	4.37
36	1.54	3.45	3.35	5.24
48	2.26	3.90	3.45	5.01
60	1.29	2.45	2.14	2.45
72	1.41	4.38	3.99	5.85
96	1.42	2.85	3.29	6.40

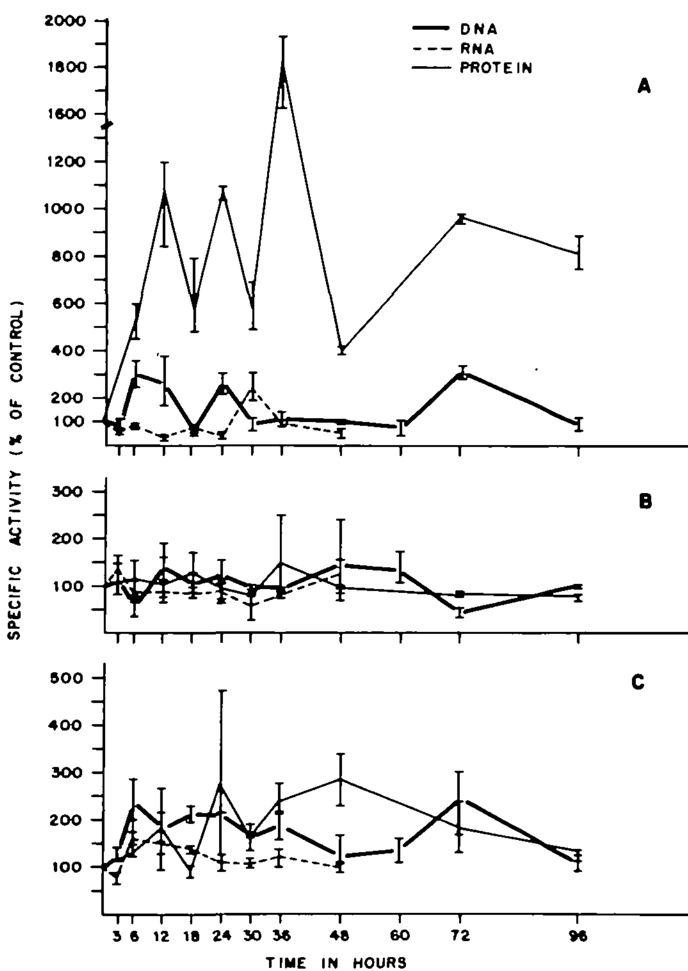

Fig. 2. The rates of labelled precursor incorporation into RNA, DNA and protein in the epidermis of mouse skin treated with (A) MCA; (B) MCA + acetone; and (C) MCA + TPA expressed as percentage specific activity of the respective control. Control animals were treated with (A) TFB; (B) MCA only; (C) MCA + acetone. Bars represent the range. Mice were given 75 μCi of [^{3}H]uridine, 33 μCi of [^{3}H]methylthymidine or 5 μCi of [^{14}C]L-leucine i.p. 1 h prior to sacrifice. RNA, - - - - -; DNA, ______; protein, ——.

creases the rate of precursor incorporation into protein at 36 h (151% of the control) the values remaining close to control at all other time points (Figure 2B). A single application

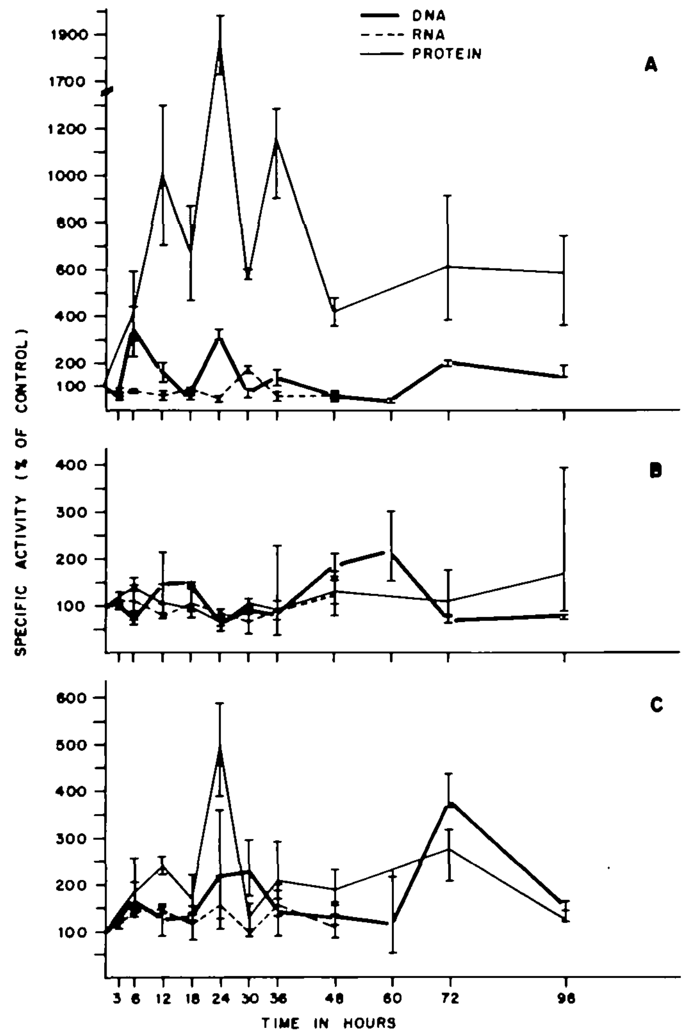

Fig. 3. The rates of labelled precursor incorporation into RNA, DNA and protein in the dermis of mouse skin treated with (A) MCA; (B) MCA + acetone and (C) MCA + TPA; expressed as percentage specific activity of the respective control. For details see the legend to Figure 2.

of TPA stimulates the rate of incorporation of [^{14}C]L-leucine into protein giving rise to 3 peaks at 12, 24, and 48 h (185%, 272% and 286% of the control, respectively) followed by a shoulder and return to near control level at 96 h.

Dermis

As seen in Figure 3A, the rate of incorporation of [^{3}H]uridine into RNA does not deviate appreciably from the control. A single peak of stimulated precursor incorporation is seen at 30 h (180% of the control). Acetone application has little impact on the rate of precursor incorporation into RNA (Figure 3B). A single TPA application causes an early stimulation of incorporation of [^{3}H]uridine into RNA at 3 h, the levels remaining slightly above the control up to 12 h. Two peaks of stimulated precursor incorporation are seen at 24 h and 36 h (153% and 158% of the control), the level reverting to control value at 48 h.

The rate of incorporation of [^{3}H]methylthymidine into DNA is elevated substantially in the precancerous dermis (Figure 3A). Following a small initial inhibition at 3 h, three peaks of stimulated incorporation appear at 6, 24, and 72 h (350%, 329% and 202% of the control, respectively). Acetone application inhibits the precursor incorporation at

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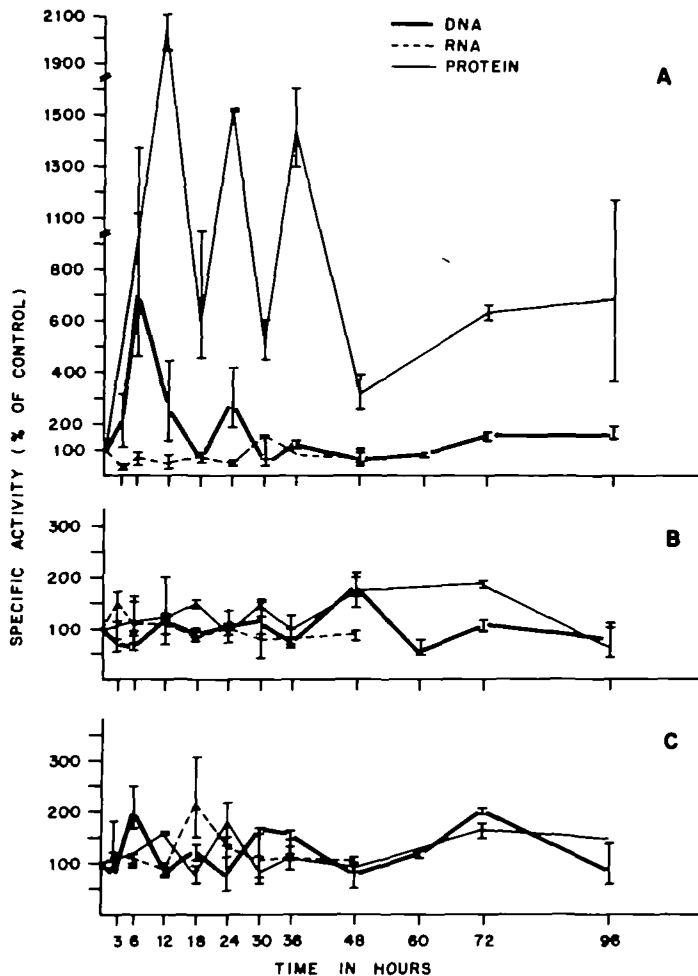

Fig. 4. The rates of labelled precursor incorporation into RNA, DNA and protein in the subcutis of mouse skin treated with (A) MCA; (B) MCA + acetone and (C) MCA + TPA; expressed as percentage specific activity of the respective control. For details see the lenged to Figure 2.

6 h, which then shows stimulation between 12 and 18 h (147% of the control). The levels remain near control values between 24 and 36 h and rise thereafter to produce a stimulated plateau between 48 and 60 h (186–216% of the control, Figure 3B). The rate of precursor incorporation into DNA declines after 60 h and is below the control values between 72 and 96 h. Animals treated with TPA (Figure 3C) show an early increase in the precursor uptake into DNA at 3 h and a small peak at 6 h (169% of the control). The activity increases between 24 and 30 h (217–229% of the control) declining thereafter to levels which remain above the control up to 60 h. Maximum stimulation of precursor incorporation is observed as a peak at 72 h (374% of the control). The incorporation rate declines thereafter to near control level at 96 h.

The rate of incorporation of [^{14}C]L-leucine into protein is markedly stimulated in the precancerous dermis (Figure 3A). A significant early increase at 6 h is followed by three sharp peaks of increased uptake at 12, 24 and 36 h (1015%, 1878% and 1155% of the control, respectively) and sustained stimulation lasting up to 96 h. Figure 3B shows that a single application of acetone to the precancerous mouse skin does not appreciably alter the rate of precursor incorporation into protein. A single TPA application rapidly increases the rate of precursor incorporation into protein which remains

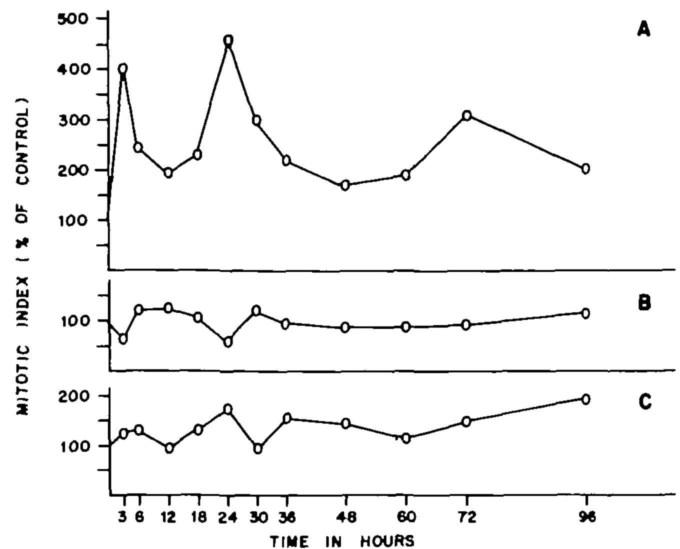

Fig. 5. Epidermal mitotic index in mouse skin treated with (A) MCA; (B) MCA + acetone; (C) MCA + TPA; expressed as percentage average mitotic index of the respective control animals treated with (A) TFB; (B) MCA only; (C) MCA + acetone.

elevated between 3 and 72 h declining thereafter to reach control level at 96 h (Figure 3C). Three peaks of stimulated precursor incorporation are observed at 12, 24 and 72 h (242%, 502% and 275% of the control, respectively).

Subcutis

As seen in Figure 4A, the rate of incorporation of [^{3}H]-uridine into RNA is slightly diminished as compared with the control between 3 and 48 h except for a small peak at 30 h (160% of the control). Acetone application elicits early stimulation of precursor incorporation into RNA at 3 h (153% of the control) and is followed by return to near control levels lasting up to 48 h (Figure 4B). TPA application results in a single peak at 18 h (221% of the control), and a gradual decline to near control level at 30 h which is maintained up to 48 h (Figure 4C).

An early stimulation of the rate of [^{3}H]methylthymidine incorporation into DNA in the precancerous subcutis at 3 h increases further giving rise to a peak at 6 h (719% of the control), the level gradually decreasing to control value at 18 h (Figure 4A). This is followed by a smaller peak appearing at 24 h (293% of the control) and a return to near control levels between 36 and 96 h. Acetone application causes an initial inhibition of precursor incorporation into DNA up to 6 h followed by return to near control levels up to 36 h (Figure 4B). A peak showing stimulated incorporation at 48 h (184% of the control) is followed by inhibition at 60 h, the levels returning to near control values around 72–96 h. As shown in Figure 4C a single application of TPA to the precancerous mouse skin produces a small initial decrease in the rate of incorporation of [^{3}H]methylthymidine into DNA at 3 h with a peak at 6 h (206% of the control). The rate decreases thereafter to remain near control value up to 24 h, and reaches a high plateau between 30 and 36 h, declining to control level at 48 h. Another peak at 72 h (205% of the control) is followed by return to control level at 96 h.

The rate of incorporation of [^{14}C]L-leucine into protein is markedly enhanced in the subcutis from precancerous mouse skin (Figure 4B). Three sharp peaks of stimulated precursor incorporation appear at 12, 24 and 36 h (2046%, 1527% and

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1441% of the control, respectively). High levels are sustained up to 96 h. Acetone application causes an initial mild increase in the rate of precursor incorporation giving rise to peaks at 18 and 30 h (151% and 149% of the control), and a plateau between 48 and 72 h, the level decreasing to control value at 96 h (Figure 4B). A single application of TPA increases the rate of precursor incorporation into protein with the first peak appearing at 12 h (162% of the control) and a second at 24 h (183% of the control). The incorporation rate remains close to control up to 48 h and increases gradually to form a plateau between 72 and 96 h (Figure 4C).

Mitotic activity

The time course of epidermal cell mitotic activity in the precancerous mouse skin as compared with solvent control is given in Figure 5A, while effects of acetone and TPA on the mitotic activity are presented in Figures 5B and 5C. All values are expressed as percentage of the respective control.

The mitotic index is elevated in the precancerous epidermis as compared with the control. An initial peak at 3 h (403% of the control) is followed by a gradual decrease to a level well above the control between 6 and 18 h and a second peak at 24 h (461% of the control). Gradual decline between 30 and 48 h gives rise to a shoulder followed by a third peak at 72 h (311% of the control), the mitotic index remaining elevated at 96 h (Figure 5A). Acetone application causes inhibition of mitotic activity at 3 and 24 h with no appreciable deviation from the control at other time points (Figure 5B). TPA application elicits an early increase in mitotic index up to 6 h which is followed by return to near control level at 12 h and subsequent increase with a peak at 24 h (175% of the control), the mitotic activity remains at a plateau between 36 and 72 h (Figure 5C) increasing to a higher level at 96 h (194% of the control).

Histology

Six weeks after a single s.c. injection of thiophene free benzene, haematoxylin and eosin stained sections of the treated skin show 1 – 2 nucleated cells thick orthokeratotic epidermis with an occasional parakeratotic area. Spindle shaped fibroblasts and thickened collagen bundles are seen in the dermis. Six weeks after MCA injection, the precancerous mouse skin shows the presence of mildly hyperplastic, 2 – 3 nucleated cells thick epidermis, and highly cellular dermis containing coarse, thickened collagen bundles. The connective tissue is edematous. A mild to moderate number of lymphocytes and polymorphonuclear leucocytes are seen in the lower dermis and subcutis. Panniculus carnosus is hypertrophied. A single application of TPA to MCA injected mouse skin shows histological changes only in the epidermis with moderate hyperplasia 4 – 5 nucleated cell layers thick, hyperkeratosis, increase in the thickness of the stratum granulosum and higher mitotic index in basal cells.

Discussion

These studies were undertaken to determine the differences in the kinetics of precursor incorporation into nucleic acids and protein in control and precancerous mouse skin constituents and to elucidate the contribution of TPA towards amplification of MCA induced epithelial and mesenchymal tumorigenesis in this tissue (7,8). Data on precancerous mouse skin constituents treated with MCA were compared with those obtained in control skin treated similarly with thiophene free benzene used as solvent for the carcinogen. The influence of TPA or acetone on the rates of nucleic acids and protein synthesis in the precancerous skin constituents were also studied. Changes in precursor incorporation reported here indicate the rate of biosynthesis of nucleic acids or protein, thus no inference is possible regarding a quantitative increase in any macromolecule.

Studies dealing with the effects of carcinogens on nucleic acids or protein synthesis in the whole mouse skin or epidermis have shown that RNA and DNA synthesis are inhibited (13 – 18). Slaga *et al.* (19) reported that a single application of a completely carcinogenic dose of DMBA causes a more protracted inhibition of DNA synthesis followed by gradual increase over the control level by six days after treatment. The rates of RNA and protein synthesis also increased. The present study was carried out at a sufficiently long interval after carcinogen administration, when the immediate toxic effects had subsided. Hence inhibition of nucleic acids synthesis was not observed in the precancerous skin constituents. However, precancerous epidermis, dermis and subcutis showed a slightly decreased rate of RNA synthesis which did not differ appreciably from the control but for a peak at 30 h. Significantly, the rate of protein synthesis was markedly elevated giving rise to sharp peaks indicating 10- to 20-fold increases in all three tissues (Figures 2A, 3A and 4A). The observed increase in the rate of protein synthesis in the absence of increase in the rate of RNA synthesis is suggestive of altered regulation of transcriptional control(s) in the precancerous skin (20). The rate of DNA synthesis also increased in the precancerous tissues as compared with that in the control. The kinetics of DNA synthesis in various skin layers suggest that carcinogen administration imposes a high degree of cell cycle synchrony as a result of which cells that have escaped differentiation enter simultaneously into DNA synthetic phase giving rise to the final peak at 72 h. A single application of acetone disturbed the cell cycle due to initial toxicity resulting in mild inhibition of DNA synthesis, whereas TPA had a stimulatory influence.

A single large dose of TPA has been shown to induce sequential stimulation of RNA, protein and DNA synthesis in the normal mouse epidermis or whole skin (2,4). This response is characteristically associated with various cell systems which are stimulated to divide and include regenerating liver after partial hepatectomy (21), phytohemagglutinin stimulated lymphocytes (22) and isoproterenol stimulated salivary gland cells (23). Using a small dose of TPA (2 nmol) comparable with that used in this study, Balmain *et al.* (24) observed that DNA synthesis is stimulated shortly after maximum stimulation of RNA synthesis in the mouse epidermis. However, no data are available regarding the rate of protein synthesis in the mouse skin or epidermis exposed to a small dose of TPA. As observed in this study, precancerous mouse skin constituents did not respond to TPA by sequential stimulation of RNA, protein and DNA synthesis. However, elevated RNA synthesis returned to control level by 48 h while major stimulation of DNA synthesis was observed at 72 h in all three skin constituents.

Many reports describe an initial inhibition of DNA synthesis followed by elevated levels in the epidermis and whole skin of normal mice exposed to a single large or small dose of TPA (4, 5, 17, 25, 26). However, the inhibitory phase was absent (2) when normal mouse skin was exposed to a second dose of TPA. A lack of initial inhibition of DNA synthesis was also observed in initiated mouse skin exposed to TPA (17). In this study early stimulation of DNA synthesis without

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an inhibitory phase was observed in all the constituents of precancerous skin and may be a characteristic response of preneoplastic cells sensitive to stimulated cell proliferation by TPA.

Balmain *et al.* (24) obtained a single peak of DNA synthesis after a major peak of RNA synthesis in the epidermis of mouse skin exposed to 2 nmol of TPA. The rate of DNA synthesis reached near control level by 24 h without further increase. In this study a single exposure of precancerous mouse skin to TPA induced early stimulation of precursor incorporation into the respective macromolecule in the epidermis, dermis and subcutis. Synthesis of nucleic acids and protein were stimulated to the same extent in the epidermis and dermis and to a lesser extent in the subcutis. However, the degree of stimulation was of a lower order than that reported in normal mouse skin exposed similarly to TPA (2,26). Smaller extent of stimulation in the subcutis may be related to the amount of TPA reaching this lowermost layer of skin. The kinetics of [^{3}H]methylthymidine incorporation into DNA in the three skin components show that up to 36–48 h after exposure, TPA induces sustained and increased proliferation of preneoplastic cell types in different layers and recruits more cells into the DNA synthetic phase. A lowered rate of DNA synthesis then ensues lasting up to 60 h which may be the result of a wave of cellular differentiation following increased mitotic activity (Figures 2C, 3C and 4C). This is followed by a peak of DNA synthesis at 72 h indicating a higher degree of synchronization of cells in the proliferative compartment. The stimulation of DNA synthesis at 72 h after TPA treatment appears to be particularly significant and truly indicative of stimulatory effect of TPA as inhibition of DNA synthesis was observed at this time point in the respective solvent control tissues.

The kinetics of epidermal mitotic activity in various groups show that the mitotic index is higher in precancerous epidermis (Figure 5A). TPA application results in a small increase in mitotic index between 3 and 6 h. This could be attributed to release of cells arrested in G2 phase (26) followed by increased levels of mitotic activity up to 96 h, and correlates well with the kinetics of DNA synthesis again indicating the increased sensitivity of precancerous cells to a small proliferative stimulus (Figure 5C).

This study shows that the precancerous epidermis, dermis and subcutis exhibit distinct characteristics such as altered translational control(s) and acquisition of cell cycle synchrony. Moreover, normal and precancerous mouse skins respond differently to a single application of TPA. The most significant contribution of TPA towards enhanced expression of neoplastic transformation appears to be its ability to accelerate selective expansion of preneoplastic cells during tumor progression.

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