Acta Dermatovener (Stockholm) 54: 343–347, 1974

THE EFFECT OF A CATIONIC TRIPHENYLMETHANE DYE (CRYSTAL VIOLET) ON RABBIT GRANULATION TISSUE

Oxygen Consumption and RNA and Protein Synthesis in Tissue Slices

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Abstract. Early metabolic reactions in granulation tissue after exposure to crystal violet for 5 minutes were studied in vitro. The tissue was taken from cylinders implanted subcutaneously in rabbits 21 days previously. The oxygen consumption rate was measured. The net synthesis of protein and collagen was estimated by the incorporation of ^{14}C-proline into proteins and into collagen hydroxyproline. The RNA synthesis was measured by the incorporation of ^{3}H-cytidine into RNA. Crystal violet at a concentration of 10 μg/ml reduced all these processes markedly, even within a few hours.

The effect of topical crystal violet (c.v.) on regenerating dermal connective tissue in the rat has previously been analysed in this laboratory. It was shown that c.v. interfered severely with tissue regeneration by increasing the acute inflammatory response and delaying the onset of fibroplasia and collagen formation (15, 16). Tissue culture studies utilizing epithelial- and fibroblast-like cells demonstrated the cytotoxic effects of c.v. and related compounds (18), but the primary target of c.v. and the subsequent events resulting in cellular dysfunction remained obscure.

The present investigation was undertaken to study some of the early biochemical effects of c.v. on regenerating tissue. The effect of the dye on the oxygen consumption capacity was measured in vitro. Its influence on the rate of RNA synthesis was measured by following the incorporation of ^{3}H-cytidine, and on that of protein and collagen synthesis by measuring the incorporation of ^{14}C-proline.

MATERIAL AND METHODS

Production of granulation tissue

Sixteen female albino rabbits weighing 2–3 kg were anaesthetized with pentobarbitone intravenously and the back was shaved. Under sterile conditions, 2 stainless steel wire mesh cylinders, 5 cm long and 2 cm in diameter, were implanted under the panniculus carnosus muscle on the flank of the rabbits as described by Holmström (6). The skin was wrapped without tension around the complete circumference of the cylinders using two pairs of stay sutures bolstered by cotton rolls (Fig. 1). Thus the cylinders were surrounded by the richly vascularized panniculus carnosus. The incision was closed by a continuous silk suture. The animals were maintained in individual cages with free access to standard pellets and water.

After 3 weeks, the cylinders were removed under light ether anaesthesia. They were immediately opened in a cold room (+4°C). Granulation tissue within the cylinders was scraped off and placed in an ice-cold incubation medium: a modified Krebs–Ringer solution was used (26) at pH 7.4 with 0.1 M Hepes as buffer (Calbiochem, Los Angeles), omitting phosphate (25) but supplemented with glucose (22.4 mM); no amino acids were added. Generally speaking, tissue from 2 animals was pooled for each set of experiments. The tissue was cut into 0.5 mm thick slices with a Stadie-Rigg microtome and weighed (wet weight).

In vitro exposure to crystal violet

Three samples of tissue, about 500 mg each, were exposed to an incubation medium with crystal violet (CI 42555; Merck, Darmstadt, BRD) added in a final concentration of 10 μg/ml (1:100 000) or 1 μg/ml (1:1 000 000) in a thermostated shaker (37°C) in air for 5 minutes. Three other tissue samples of similar weight were processed identically except that crystal violet was not present (controls). The slices were then transferred to bottles with new incubation medium without c.v., and were arranged in pairs of one dye-exposed and one control specimen. One pair of samples was analysed for oxygen consumption, one pair was used for incubation with ^{14}C-L-proline and the last pair used for incubation with ^{3}H-cytidine (Fig. 2).

Oxygen consumption

Oxygen consumption by the tissue samples was determined by a Biological Oxygen Monitor®, Model 53 (Yellow Springs

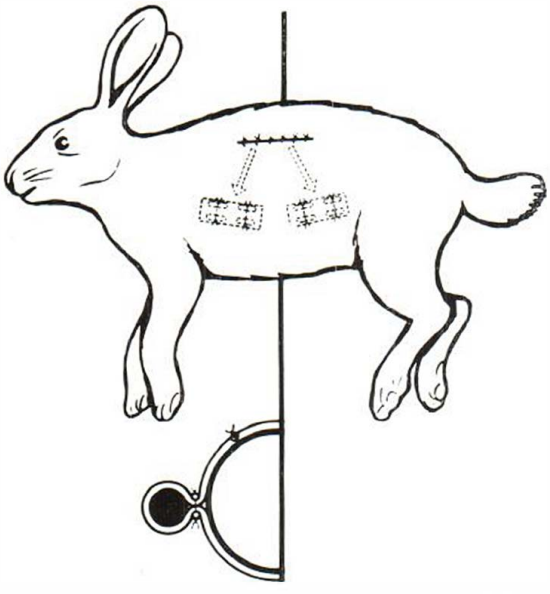

Fig. 1. Subcutaneous implantation of wire mesh cylinders. The surrounding "skin fold" was created by cotton rolls and loose sutures.

Instrument Co., Inc., Yellow Springs, Ohio, USA). It has a Clark-type polarographic oxygen electrode which measures the oxygen tension of the incubation medium in the closed sample chamber. The medium contains 5.0 μl O_2/ml at 37°C and ambient barometric pressure. Samples about 500 mg in 3 ml incubation medium were used. The respiring tissue consumes oxygen, and the decreasing oxygen tension of the sample medium was continuously monitored by a recorder.

The oxygen assays were performed at 37°C either immediately after exposure to the dye or control solution, or 30 minutes later (meanwhile placed in incubation medium at 37°C).

In a recent study by Holmström et al. (4), the oxygen consumption rate (Q_{O_2}) of granulation tissue was found to decrease slowly with lowering oxygen tension down to P_{O_2} about 20 mmHg, below which it fell rapidly. In the present study, all Q_{O_2} measurements were calculated at P_{O_2} 90 mmHg, and expressed as μl O_2/mg wet weight/hour. The assay time required to reach P_{O_2} 90 mm ranged, for control tissue, between 14 and 38 minutes, and for dye-treated tissue, between 17 and 90 minutes.

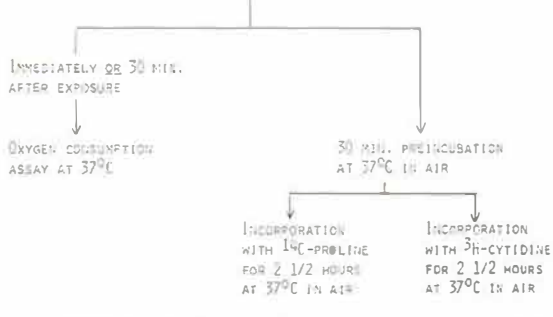

Fig. 2. Management of granulation tissue slices after exposure to crystal violet or control solution. Pairs of dye-treated and control samples were compared in each assay. Each tissue sample (approx. 0.5 g) was only used for one measurement.

Incorporation of labelled proline and cytidine in vitro

The tissue samples, each weighing approximately 500 mg, were preincubated in 3 ml incubation mixture for 30 minutes in air in a thermostated shaking bath at 37°C. Then the radioactive compounds were added: 5 μCi L-proline-C^{14} (U) (285 mCi/mmol. The Radiochemical Centre, Amersham, England) to one pair of tissue samples, and 50 μCi cytidine-5-H^3 (17 Ci/mmol. The Radiochemical Centre) to another pair of samples. The incubations continued for 2½ hours.

Measurement of ^{14}C-proline incorporation

The incorporation was stopped by adding 2 vol ethyl alcohol to the incubation mixture. The slices were collected by centrifugation in a refrigerated centrifuge, suspended in ethyl alcohol and recentrifuged. After drying the sediment to constant weight at room temperature it was homogenized with an Ultra-Turrax® homogenizer in distilled water containing unlabelled proline and dialysed against running tap water for 48 hours. The dialysed homogenate was then hydrolysed in 6 N hydrochloric acid at 130°C for 3 hours. Hydrochloric acid was removed by careful evaporation in a water bath with several washings of distilled water. The residue was dissolved in water and its total radioactivity and the radioactivity of hydroxyproline were determined as described by Juva & Prockop (10). The recovery of hydroxyproline was 25–35%. The results were expressed as dpm/mg dry weight.

Measurement of ^{3}H-cytidine incorporation

The incorporation was terminated by adding 1.0 N perchloric acid (PCA) to a final concentration of 0.5 N in an ice-bath. For the extraction of total RNA a modified Schmidt–Thannhauser procedure was used (22). The slices were homogenized in an ice-bath and the homogenate centrifuged at 20 000 *g* for 40 minutes at 0°C. The pellet containing RNA, DNA and tissue proteins was washed twice with ice-cold 0.5 N PCA. RNA was then hydrolysed to component mononucleotides by incubating at 37°C for 16 hours in 0.3 N potassium hydroxide. The solution was then cooled in an ice-bath and neutralized with ice-cold 3 N PCA to pH 1–2. Precipitated DNA and proteins were removed by centrifugation at 20 000 *g* for 40 minutes at 0°C. The supernatants were used to measure the radioactivity and RNA ribose (3).

Measurement of radioactivity

A Packard Tri-Carb liquid scintillation spectrometer was used. ^{3}H-hexadecane or ^{14}C-toluene was used as an internal standard to measure counting efficiencies. Aqueous solutions were counted in a commercial scintillation mixture, Insta-gel® (Packard Instrument Co., USA).

Table I. *Effect of crystal violet on the oxygen consumption rate of slices of granulation tissue from subcutaneously implanted cylinders*

Exp. no.	Crystal violet conc.	Interval dye exposure–O_2 analysis (min)	Q_{O_2} μl O_2/mg wet weight/hr		Q_{O_2} ratio[a] (%)
			Control	Test	
1[b]	1 : 10^5	Immediate	0.025	0.024	96
2[b]	1 : 10^5	Immediate	0.027	0.023	85
3	1 : 10^5	Immediate	0.019	0.013	68
4[b]	1 : 10^5	30	0.024	0.008	33
5	1 : 10^5	30	0.043	0.018	42
6	1 : 10^5	30	0.053	0.026	49
7	1 : 10^5	30	0.032	0.016	50
8[b]	1 : 10^6	30	0.033	0.042	127
9[b]	1 : 10^6	30	0.022	0.022	100
10[b]	1 : 10^6	30	0.037	0.026	70

[a] $\frac{\text{test value}}{\text{control value}} \times 100$.

[b] Samples from the same tissue pool also analysed for radioisotope incorporations.

RESULTS

Oxygen consumption

Table I shows the results of the oxygen consumption measurements. Sliced granulation tissue exposed to c.v. 10 μg/ml for 5 minutes showed a slight reduction in its oxygen consumption rate as compared with controls when the oxygen assay started immediately. In samples treated similarly but with the oxygen consumption measurements started 30 minutes later, the oxygen consumption rate was pronouncedly lower than in corresponding control samples. When c.v. 1 μg/ml was used and the oxygen assay also started 30 minutes after the tissue had been exposed, no consistent change in the oxygen consumption rates was recorded.

^{14}C-proline incorporation

The incorporation of ^{14}C-proline into proteins (total radioactivity) of granulation tissue was markedly reduced after in vitro exposure to c.v. 10 μg/ml (Table II). With c.v. 1 μg/ml the incorporation was lower than in control samples, but the difference was not as great as at the higher dye concentration.

The formation of ^{14}C-hydroxyproline, taken as a measure of the capacity to synthesize collagen, was severely decreased in slices of granulation tissue treated with c.v. 10 μg/ml (Table III). The difference was smaller with c.v. 1 μg/ml.

Table II. *Overall incorporation of ^{14}C-proline as such in slices of granulation tissue following exposure to crystal violet*

Exp. no.	Crystal violet conc.	Total ^{14}C of protein hydrolysate (dpm/mg dry weight)		Ratio[a] (%)
		Control	Test	
1	1 : 10^5	13 937	3 703	27
2	1 : 10^5	2 373	325	14
4	1 : 10^5	7 319	532	7
8	1 : 10^6	5 131	1 226	24
9	1 : 10^6	5 549	3 886	70
10	1 : 10^6	2 145	1 492	70

[a] $\frac{\text{test value}}{\text{control value}} \times 100$.

^{3}H-cytidine incorporation

The rate of RNA formation, as judged from the incorporation of ^{3}H-cytidine into RNA, was markedly depressed in granulation tissue treated with c.v. 10 μg/ml (Table IV). The lower concentration of c.v. 1 μg/ml had no clear effect.

DISCUSSION

Subcutaneous implantation of cylinders starts a healing process with growth of granulation tissue into the cylinder space (7, 21). The newly formed tissue, which can readily be separated from adjacent old tissue, is free from foreign body reaction (5, 21), as distinguished from tissue in sponge implants (16). The composition of the tissue within the cylinder at different stages of development has recently been

Table III. *Incorporation of ^{14}C-proline into collagen hydroxyproline by slices of granulation tissue following exposure to crystal violet*

Exp. no.	Crystal violet conc.	^{14}C-hydroxyproline (dpm/mg dry weight)		Ratio[a] (%)
		Control	Test	
1	1 : 10^5	1 023	214	21
2	1 : 10^5	246	30	12
4	1 : 10^5	905	58	6
8	1 : 10^6	197	48	24
9	1 : 10^6	220	219	100
10	1 : 10^6	85	42	49

[a] $\frac{\text{test value}}{\text{control value}} \times 100$.

Table IV. *Effect of crystal violet on the incorporation of ^{3}H-cytidine into RNA in slices of granulation tissue*

Exp. no.	Crystal violet conc.	^{3}H-cytidine, cpm / μg of RNA-ribose		Ratio[a] (%)
		Control	Test	
1	$1:10^5$	27 436	3 931	14
2	$1:10^5$	19 613	4 925	25
4	$1:10^5$	20 215	4 206	21
8	$1:10^6$	14 257	20 444	143
9	$1:10^6$	14 457	11 392	79
10	$1:10^6$	15 211	13 385	89

[a] $\frac{\text{test value}}{\text{control value}} \times 100$.

characterized biochemically and histologically in a series of experiments by Holmström and co-workers (6). Granulation tissue obtained 3 weeks after implantation was chosen for the present experiment since it has a high metabolic activity with high capacities for oxygen consumption and protein synthesis. Slices from similar tissue can be kept respiring in an incubation medium for more than 6 hours (1, 11).

The incubation medium used in this study contains oxygen and glucose in excess of the amounts found in the healing tissue within the cylinder (6). Therefore, the in vitro assays test the capacity of the tissue to synthesize proteins and RNA, rather than reflecting the situation in vivo. It is, however, the relative difference between dye-exposed and control tissue that is of interest.

Specific activity of labelled proline has not been measured. Increased catabolism might reduce specific activity and result in reduced ^{14}C-proline incorporation. It cannot be concluded that crystal violet has changed protein synthesis alone, since changes in catabolism might also occur. However, the results indicate that crystal violet reduces net protein synthesis (synthesis-degradation).

The present experiments demonstrated that crystal violet (c.v.) alters tissue metabolism in vitro. Tissue exposure for 5 minutes to a concentration of 10 μg/ml resulted in a reduction of the relative oxygen consumption rate and reduced capacities for the synthesis of RNA and the incorporation of ^{14}C-proline. Exposure to a lower concentration, 1 μg/ml, had no definite effect. This lower concentration significantly impaired the multiplication of fibroblast-like cells in tissue culture (18). This discrepancy can be explained as a greater sensitivity of single cells to the influence of various substances than applies to cells in a composite tissue (20).

C.v. thus triggers a sequence of metabolic reactions, but its primary point(s) of attack is obscure. With c.v. (10 μg/ml) the oxygen consumption rate of the granulation tissue was markedly reduced when the oxygen assay started 30 minutes after the end of tissue exposure to the dye. It is interesting that this effect was only slight when the assay was started directly after dye exposure. The penetration rate of c.v. into the tissue may be important here—microscopically there is a pronounced interstitial metachromasia, indicating the presence of acid mucopolysaccharides (5), which in a preceding study reduced the cytotoxicity of c.v., probably by binding the polyanionic tissue components to the cationic dye (18).

Oxygen is essential for energy production, collagen synthesis (hydroxylation of proline), and cell proliferation in regenerating tissue (8). Reasonable explanations of the decreased tissue respiration following exposure to c.v. could be either a primary effect of the dye on the energy metabolism being dominant in these cells (glucose intermediary metabolism and mitochondrial function), or an adaptation to smaller energy demands resulting from decreased cytoplasmic protein synthesis.

The literature supports an affinity of c.v. to nucleic acids. C.v. has been used as a nuclear or chromatin stain (14); and a binding to DNA has been demonstrated by cytophotometry (17). This might induce detrimental changes in the DNA molecules (19). With this in mind, an influence on cells during the period of DNA synthesis is to be expected. Recently, however, Norrby & Mobacken (18) showed that not only these but also cells in the G1 (0) cell cycle phase are influenced by c.v. This indicates that c.v., besides influencing nuclear DNA, also influences other basic mechanisms in living cells.

Mitochondria contain DNA which is believed to code the synthesis of some mitochondrial proteins (2, 12). Some chemicals are known to interact with mitochondrial DNA and thus disturb the proper functioning of mitochondria. Ditranol, a common topical antipsoriatic drug, as also acridines, is proposed to act thus, probably by intercalation (13, 24, 27). An induction of respiration-deficient mutants of yeast with c.v. in concentrations of $10^{-7}-10^{-6}$ g/ml has recently been shown (23). This type of mutation

is caused by interaction with the mitochondrial DNA. Possible mechanisms for interaction with DNA have recently been reviewed by Irving (9). Other substances block respiratory enzyme systems, e.g. rotenone, antimycin A and cyanide, and cause a quick inhibition of respiration (12). A reduced respiratory activity, irrespective of its cause, will result in a slowing of cellular functions.

It is also possible that c.v. acts primarily on the nucleic acids in cell nuclei and/or cytoplasmic RNA with secondary effects on cytoplasmic protein synthesis, which in turn will reduce the requirement for energy and thus limit mitochondrial respiration. A combination of the discussed targets for c.v. cannot be ruled out.

In conclusion: this study has shown that c.v. seriously impairs the metabolism of connective tissue cells in vitro. Several pathogenetic mechanisms are possible, as the primary event(s) is still unknown. Further studies are necessary for a definite clarification of the mode of action of c.v. Furthermore, our observations suggest that the in vitro system employed in this study may be a useful model for the study of the metabolic effects of topical drugs on granulation tissue.

ACKNOWLEDGEMENTS

This study was supported by grants from Göteborgs Läkaresällskap and the Swedish Medical Research Council (B74-17X-640-10B). Technical assistance rendered by Monica Olsson and Ulla-Britt Carlsson is gratefully acknowledged.

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Received February 7, 1974

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