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Activation of Lysosomal Enzymes and Tumour Regression Caused by Irradiation and Steroid Hormones*

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Abstract—*The lysosomal enzyme activity and membrane permeability of mouse C3H mammary tumours has been studied using quantitative cytochemical methods following irradiation of the tumours with doses of* 1500, 3500 *or* 6000 rad *γ rays. No change in the lysosomal enzyme activity was observed immediately after irradiation, but increased enzyme activity and increased membrane permeability were observed* 24 hr *after irradiation with doses of* 3500 *or* 6000 rad. *Twenty-four hours after injection of prednisolone there was a marked increase of lysosomal membrane permeability and enzyme activity, and injection of prednisolone soon after irradiation enhanced the effect of irradiation. After a dose of* 6000 rad *and prednisolone, the lysosomal membrane permeability increased to* 191% *of the control and the enzyme activity to* 326% *of the value of the control tumours. Measurement of tumour size after irradiation or after a combined treatment with irradiation and prednisolone showed that a close correlation exists between tumour regression and lysosomal enzyme activity. The experiments support the view that lysosomal enzymes play an important role in tumour regression following irradiation.*

INTRODUCTION

It is well established that lysosomes play an important role in tissue involution, as has been demonstrated for many tissues such as the uterus [1] and the mammary gland [2]. During spontaneous regression of the Jensen sarcoma, increased activity of several lysosomal hydrolases has been demonstrated [3].

Several steroid hormones have also been shown to affect the stability of lysosomal membranes. Hydrocortisone protects lysosomes in suspension against labilising agents *in vitro* [4, 5], but injections of prednisolone and hydrocortisone exert a powerful labilising effect on lysosomes of spleen and thymus [6, 7].

Tumour involution caused by irradiation or drug therapy may ultimately also be a result of enhanced activity of lysosomes and of lysosomal enzymes and, using ultra-centrifugation techniques, it has been demonstrated that irradiation causes release of hydrolases from lysosomes of mammary tumours into the supernatant [8, 9].

More sophisticated quantitative cytochemical methods have been used to show that lysosomal enzyme activity in HeLa cells is strongly increased by low doses of irradiation [10], and in mouse mammary tumours following whole-body irradiation [11].

These experiments have shown that irradiation can cause lysosomal activation, but the possibility that there is a direct correlation between activation of lysosomal enzymes and tumour regression has not been previously investigated. In this paper we describe the relationships between lysosomal enzyme activation and tumour regression caused by irradiation and injection of steroids.

MATERIALS AND METHODS

Tumour-bearing mice

Spontaneous primary adenocarcinomas arising in the abdominal position in C3H Bart's female mice inbred and kindly provided by the Department of Radiobiology, St. Bartholomew's Hospital Medical College, were used for some experiments. The mice were aged between 16 and 24 months and weighed between 35 and 40 g.

For the majority of experiments the tumours were transplanted into male C3H mice. A section of tumour tissue, approximately 27 mm^3, was removed from a female mouse, inserted

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into an incision in the thigh of a male mouse weighing 30–35 g and the incision closed. The majority of mice developed tumours which were used after 1–3 months, when they had grown to 500–1000 mm^3. Groups of 4 mice were used in most experiments.

Tumour sizes were measured daily in 3 dimensions using accurate micrometer calipers. The 3 measurements were multiplied to give a 'relative tumour volume' [12].

Irradiations

Irradiation was carried out using a 1500 C' source of [^{60}Co] rays. The dose rate was 350–400 rad/min and doses of 1500, 3500 or 6000 rad were given directly to the tumours. For irradiation, the mice were anaesthetised with 'Sagital' (40 mg/kg) and placed in perspex boxes of sufficient size to contain the mice and with adequate air vents. A lead block 5 cm thick with a hole 1–2.5 cm diameter was fixed to one side of the box and the hole adjusted exactly over the tumour. The shielding reduced the dose given to the remainder of the body to 3% of that received by the tumour. Doses were checked by irradiation of ferrous sulphate solutions and by using a Baldwin–Farmer secondary standard dose meter.

Control mice were also anaesthetised with Sagital (40 mg/kg) but were not irradiated.

Measurement of lysosomal acid phosphatase activity

Acid phosphatase was chosen as a typical lysosomal enzyme for study and the activity was measured by the quantitative cytochemical procedure, described in detail by Aikman and Wills [6] and Clarke and Wills [11].

For this method, the tumours were dissected out and slices of tumour 2–3 mm thick were rapidly frozen in a bath of hexane kept at −60 to −70°C. Slices 7 μm thick were then cut in a cryostat at −40°C and transferred to glass slides. Slices were then incubated for periods of up to 90 min in a medium containing acetate buffer (pH 5.0) lead nitrate and sodium β-glycerphosphate as substrate. Slices were removed at intervals, rinsed and treated with a saturated solution of hydrogen sulphide. After mounting, the absorbance of the lead sulphide stain produced was measured using a M85 Vickers Scanning and Integrating Microdensitometer. Slides were examined at a magnification of ×40 using mask size A, which gave a scanning area of 20 μm^2, and spot size 1 was used for all measurements.

For each experiment 5 groups of 4 slides were measured, one group being incubated with sodium fluoride to inhibit acid phosphatase so that the mean value of the readings obtained gave a blank value. The other 4 groups were incubated for 0, 20, 40 or 60 min in the substrate medium. Ten areas from each slide were scanned using the microdensitometer and the mean absorbance reading of all areas calculated for each group, or time of incubation.

The net mean absorbance reading for each mouse at each incubation time was calculated to give an average value for the enzyme activity of all animals used in the experiment. The values calculated were plotted against incubation time. Rates were measured by regression line analysis.

Earlier investigations on lysosomal enzyme activity using quantitative cytochemical methods [6, 7, 10] showed that the rate of increase of absorbance with time usually showed a point of inflection after 15–20 min incubation. There is good evidence in support of the concept that the rate measured over the initial period of incubation (0–20 min) gives a measure of the permeability of the lysosomal membrane to the substrate, and the subsequent rate measured over 20–60 min gives a measure of the enzyme activity.

RESULTS

Effect of irradiation on lysosomal enzyme activity in tumours

Transplanted tumours in a group of 4 male mice were irradiated with 3500 rad.

No change in lysosomal enzyme activity was observed in the tumour sections immediately after irradiation, but 24 hr after irradiation a marked increase was observed, especially during the initial (0–20 min) period of incubation of the sections in the substrate medium (Table1).

Irradiation also caused activation of lysosomal acid phosphatase in spontaneous tumours in female mice. A dose of 6000 rad caused the initial (0–20 min) rate of acid phosphatase action to increase to 142 ± 19% of the control value 24 hr after the irradiation and to 174 ± 35% of the control 48 hr after irradiation.

Effect of steroid hormones on lysosomal acid phosphatase activity

In view of the fact that several steroid hormones can markedly affect the stability of the lysosomal membrane [3, 4, 6], experiments were carried out to study the effects of some steroid hormones on lysosomes of tumours and to establish whether they would stabilise the

Table 1. *Lysosomal acid phosphatase activity in tumours after irradiation*

Incubation time	Enzyme activity (% untreated control) After 1 hr	After 24 hr
0–20 min	103 ± 10 (NS)	162 ± 14 ($P < 0.001$)
20–60 min	100 ± 14 (NS)	122 ± 33 (NS)

Groups of mice were given a dose of 3500 rad to the tumour and acid phosphatase activity was determined 1 or 24 hr later.

Rates are expressed as % of untreated controls ± S.E.M. *P* values are shown for a two-sample *t*-test in comparison with untreated controls.

Table 2. *Lysosomal acid phosphatase activity in tumours after treatment with prednisolone*

Incubation time;	Enzyme activity (% untreated control)
0–20 min	182 ± 13 ($P < 0.001$)
20–60	99 ± 10 (NS)

Groups of mice were injected with 2 mg prednisolone intraperitoneally and acid phosphatase activity was determined 24 hr later.

Rates are expressed as % of untreated controls ± S.E.M. *P* values are shown for a two-sample *t*-test in comparison with untreated controls.

lysosomes or would enhance the labilising effects of radiation.

Groups of male mice possessing transplanted tumours were injected with doses of 50 or 100 mg cortisone acetate/kg body weight. Tumour sections were prepared 1 and 24 hr after injection for determination of acid phosphatase activity.

One hour after injection the lysosomal enzyme activity, measured as the initial rate, was reduced to 70–80% of the control value, indicating that stabilisation of the lysosomal membrane had occurred, but after 24 hr the enzyme activity returned to values close to those of the control. Stabilisation of the lysosomes also resulted from hydrocortisone treatment, which caused the enzyme activity to drop to 68 ± 18% of the control, and this was maintained for 24 hr.

In contrast, injection of male mice bearing transplanted tumours with prednisolone (2 mg) caused a marked enhancement of lysosomal enzyme activity 24 hr after injection (Table 2). This steroid had a very significant effect on the initial rate of enzyme activity (0–20 min), which was increased to 182 ± 13% of the control, whereas the subsequent rate (20 ± 60 min) was little affected. These findings indicate that the hormone had greatly increased the permeability of the lysosomal membrane to the substrate but had not affected the enzyme activity.

Effect of combination treatment of tumour with prednisolone and irradiation

The experiments described showed that, of the steroids tested, prednisolone was the only effective labiliser of lysosomal membranes. Therefore this hormone was studied in conjunction with irradation in an attempt to establish whether the labilising effect of irradiation could be increased by steroid treatment.

Tumours of a group of male mice were irradiated with 6000 rad and 90 min later the mice were injected intraperitoneally with 2 mg prednisolone. They were killed 24 or 48 hr after the injection and measurement of acid phosphatase activity was made on sections of tumour. This treatment caused a large increase in lysosomal enzyme activity 24 hr after irradiation, which was maintained for at least 48 hr (Table 3). Increases were observed both during the initial period of incubation (0–20 min) and during the subsequent period (20–60 min) of incubation.

If the rates of enzyme action over the first 20 min of incubation and for the 20–60 min incubation shown in Table 3 are compared with those for prednisolone treatment alone (Table 2), it will be noted that the rates measured over the first 20 min, 182 and 191% of the controls, were not significantly different in the two experiments. Prednisolone alone did not alter the enzyme activity measured over 20–60 min, but this was increased to 326% of the control by irradiation. It would therefore appear that prednisolone and irradiation may act synergistically on the activation of lysosomes, prednisolone by increasing the membrane permeability, and irradiation by increasing the enzyme activity.

Table 3. Lysosomal acid phosphatase activity in tumours after treatment with irradiation and prednisolone

Incubation time	Enzyme activity(% untreated control) After 24 hr	After 48 hr
0–20 min	191 ± 37 ($P < 0.05$)	181 ± 25 ($P < 0.002$)
20–60 min	326 ± 35 ($P < 0.001$)	285 ± 87 ($P < 0.01$)

Groups of mice were given a dose of 6000 rad to the tumour followed by prednisolone (2 mg) 90 min later. Acid phosphatase activity was determined 24 hr and 48 hr after irradiation.

Rates are expressed as % of untreated controls ± S.E.M. *P* values are for a two-sample *t*-test in comparison with untreated controls.

Relation between lysosomal enzyme activity and tumour growth after irradiation

For studies of tumour growth, spontaneous tumours growing under natural conditions were used. Progress of tumour growth was studied by measurements made as described, initially at daily intervals for a period of 7–10 days and then at twice-weekly intervals for a maximum of 60 days, when the tumour had grown, on average, to 400% of the original volume.

For the study of the relationship of lysosomal enzyme activity to tumour regression, groups of female mice bearing spontaneous tumours were treated with doses of 1500 rad or 6000 rad given directly to the tumours. A third group of mice bearing tumours was injected with prednisolone (2 mg) 90 min after receiving a dose of 6000 rad to the tumours, and a fourth group was treated with prednisolone (2 mg) but were not irradiated. Volumes of the tumours were calculated from measurement of 3 orthogonal diameters made on the day of the injection or irradiation and on subsequent days. Twenty-four and 48 hr after irradiation, tumours were removed, measured and the lysosomal acid phosphatase activity determined as described.

The mean values of the treated tumours, expressed in relation to the volumes of control tumour, were plotted against lysosomal acid phosphatase activity which was also calculated as a percentage of the control rate (Fig. 1).

Twenty-four hours after a dose of 1500 rad, a very small reduction in the size of the tumours was observed and this was accompanied by a decrease in lysosomal enzyme activity during the initial period of incubation.

Twenty-four hours after a dose of 6000 rad, however, a large increase in lysosomal enzyme activity occurred which was accompanied by a decrease in tumour volume, and 48 hr after the dose, the tumour volume further decreased and the enzyme activity increased.

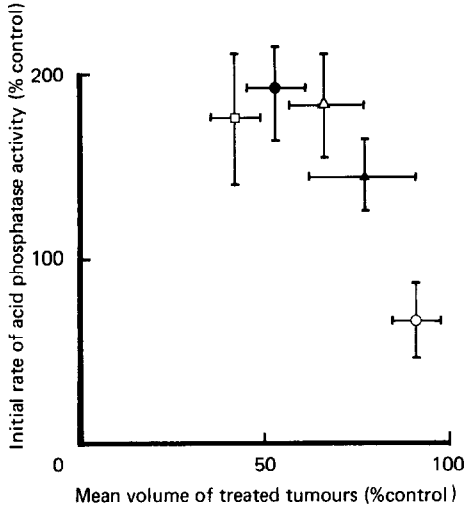

Fig. 1. The relationship between tumour volume and acid phosphatase activity after local irradiation. Tumour volumes are mean values calculated as a percentage of the volumes of untreated tumours 24 *or* 48 hr *after the treatment.* ○ 24 hr *after* 1500 rad; ▲ 24 hr *after* 6000 rad; □ 48 hr *after* 6000 rad; △ 48 hr *after* 2 mg *prednisolone*; ● 24 hr *after* 6000 rad + 2 mg *prednisolone.*

After each treatment a close relationship was apparent between increases in lysosomal enzyme activity and tumour regression.

The effect of prednisolone treatment alone and combined with irradiation was also studied over longer periods. Twenty-five days after prednisolone injection, the mean volume of a group of tumours was only 70 ± 8% of the initial volume, whilst during this period the untreated group of tumours had grown to 252 ± 16% of their initial volume. After 35 days, however, the treated tumours commenced to grow at a rate which was very similar to that of the controls.

Ten days after treatment with prednisolone (2 mg) and irradiation (6000 rad), the mean tumour volume had decreased to approximately 50% of the initial mean volume.

The growth of transplanted tumours was also strongly inhibited by prednisolone injection. Three to five weeks after the injection of prednisolone (2 mg), the mean volumes of the treated group of tumours had increased to 125% of the initial volume, whereas the volume of the untreated group had increased to 440% of their mean initial volume.

One week after treatment with prednisolone (2 mg) and irradiation (6000 rad), the mean volume of transplanted tumour had decreased to 32% of the initial mean volume, but the untreated group had grown to 190% of their initial volume.

The effects of the prednisolone and irradiation on transplanted tumours are therefore similar to those observed for spontaneous tumours and it is thus apparent that treatments which cause a stimulation of lysosomal enzyme activity can cause a pronounced regression of tumour size or delay in tumour growth.

DISCUSSION

In a previous publication from this laboratory we described increases in lysosomal acid phosphatase and β-naphthylamidase activities in mouse mammary tumours 24 hr, 48 hr and 72 hr after whole-body doses of 3600 rad [10]. We have now applied doses of 1500–6000 rad to the tumour directly whilst the remainder of the body was shielded, and have observed marked activation of lysosomal acid phosphatase 24 and 48 hr after doses of 3500 or 6000 rad. These experiments therefore demonstrate that the lysosomal enzyme activation observed is most likely to be a direct consequence of irradiation on the tumour cells and not caused indirectly by effects on other tissues, for example by alterations in hormonal control. It is noteworthy that a time interval of 24 hr was essential before lysosomal enzyme activation was observed (Table 1), and it is thus likely that some metabolic or deteriorative changes in the cells must take place before activation of lysosomal enzymes occurs. Direct effects of irradiation on the lysosomes appears unlikely because these changes would have been observed earlier, and it was suggested previously [11] that other cell organelles such as the nucleus might be involved in the initial damage.

Of the steroid hormones tested, prednisolone, hydrocortisone and cortisone, only prednisolone caused labilisation of the lysosomes and increased lysosomal enzyme activity. Hydrocortisone and cortisone were stabilisers. The effect of prednisolone was confined almost entirely to increasing the initial rate and thus is likely to cause an increase in lysosomal membrane permeability. It may be significant that prednisolone was found to be the most effective steroid in causing labilisation of lymphoid tissue lysosomes [7].

A combination of prednisolone and irradiation treatments caused a very large increase in both the initial (0–20 min) and final (20–60) rates (Table 3). The increase in the initial rate is not, however, greater than that caused by prednisolone alone (Table 2). It is therefore likely that the hormone has caused the lysosomal membranes to become fully permeable to the substrate, and little further increase is possible. Irradiation does, however, cause a very large increase in lysosomal enzyme activity, as shown by the increase to 326% of the control volume for the final rate (Table 3). This may be a result of cellular constituents partially degraded as a result of irradiation entering the lysosome to undergo hydrolysis. The combination of steroid and irradiation is thus very effective in stimulating overall lysosomal enzyme activity.

It is significant that tumour regression always appears to be accompanied by increased activity of lysosomal enzymes, and although an exact correlation cannot yet be demonstrated, the two measurements are clearly closely related (Fig. 1). This relationship is shown after irradiation treatment alone or after a combined treatment with prednisolone and irradiation which together cause a very large increase in lysosomal enzyme activity.

The results therefore provide strong evidence for an important role of lysosomal enzymes in tumour regression. The enzyme studied in the current investigation, acid phosphatase, may be regarded as a typical lysosomal enzyme and it is likely that during its activation many other enzymes of the lysosomes become activated. Enzymes such as the cathepsins, hydrolysing proteins and nucleases hydrolysing nucleic acids are likely to be of major importance because their activation will lead to disintegration of vital cell proteins and nucleic acids and thus to cell death.

Labilisation of lysosomes may thus be an important aspect of tumour therapy by ionising radiation, and lysosome labilising agents, such as certain steroids, could be useful synergists in radiation treatment.

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