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RADIATION SENSITIZING EFFECT OF DIAMIDE ON HUMAN CELLS CULTIVATED IN VITRO

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Diazenedicarboxylic acid bis (N,N-dimethylamide), diamide, oxidises intracellular non-protein SH (NPSH) specifically (KOSOWER et coll. 1969). This finding led to the assumption that diamide might be a more efficient sensitizer of anoxic and extremely hypoxic cells to radiation than of aerobic cells (RÉVÉSZ et coll. 1963, HARRIS et coll. 1969).

This was found to be true for Pseudomonas fluorescence and for 3 Chinese hamster cell lines (V79, CHO and B14AF) cultured in vitro (HARRIS & POWER 1973). However, for a high concentration (200 μM) a low sensitizing effect was observed also for aerobic V79-cells. The sensitizing effect by diamide on extremely hypoxic cells was reported by these authors to involve a reduction or removal of the shoulder of the dose-survival curve without any effect upon the slope of the exponential portion of the curve. This would give rise to a relatively efficient enhancement of radiation injury for low doses, in contrast to that found for the majority of other sensitizers, which commonly have little effect on the shoulder region, whereas the slope of the survival curve is increased for higher doses (PARKER et coll. 1969, RÉVÉSZ & LITTBRAND 1970, ADAMS et coll. 1971, CHAPMAN et coll. 1971, 1972, PETTERSEN et coll. 1973, 1974a). Therefore HARRIS & POWER suggested that enhanced cell damage by a given dose may be obtained by combining diamide with a slope-modifying sensitizer.

The ability of diamide to oxidise glutathione is reversed in the presence of glucose

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HARRIS et coll. 1971, HARRIS & BIAGLOW 1972). HARRIS & POWER gave survival data only for cells irradiated in absence of glucose. Thus, from a therapeutic point of view it would be of interest to investigate whether or not diamide acts as a sensitizer of hypoxic mammalian cells when glucose is present.

HARRIS et coll. (1974), using mouse tumours in vivo, found that the loss of non-protein SH in vivo was only 25 per cent of that expected from in vitro data for a glucose-free medium. The diamide concentration was approximately 1.7 mM within the tumour. The reduced loss of non-protein SH in vivo was ascribed to the presence of glucose. In spite of this finding a high dose-modifying sensitizing effect was observed on hypoxic tumours while there was no effect on aerobic tumours. This indicates that the concentration of NPSH may not be directly correlated with sensitivity to radiation, and therefore that the sensitizing effect of diamide may in some complicated way depend on the glucose concentration.

The modifying effects of diamide (of various concentrations) on cells irradiated in growth medium, containing about 1 mg/ml glucose was experimentally investigated. This glucose concentration is close to that of human blood. The results are discussed in the light of the results presented by HARRIS & POWER and WATTS et coll. (1975).

Materials and Methods

The experimental set-up and techniques used for irradiation of aerobic and extremely hypoxic cells have been described previously (PETTERSEN et coll. 1973). The irradiation was performed at room temperature with asynchronous populations of cells suspended in a mixture of growth medium E2a 75 %, and trypsin solution 25 % (PUCK et coll. 1957).

Extremely hypoxic cells were obtained by a degassing procedure with a gas mixture of N_2 97 % and CO_2 3 % containing less than 4 ppm O_2. During degassing and irradiation (the degassing continued during irradiation) the cell suspension (max. 20 ml) was kept in a Petri dish placed in a stainless steel chamber and was continuously agitated by a magnetic stirrer in the chamber. A hypodermic needle mounted in the chamber wall (PETTERSEN et coll. 1973) allowed samples to be extracted and diamide added without exposing the suspension to air.

The toxic effect of diamide on reproduction of aerobic cells at room temperature was tested by exposing suspended cells to diamide at different concentrations for 2 hours. The toxic effect of diamide on reproduction of extremely hypoxic cells was tested by adding diamide to a cell suspension deoxygenated by N_2 flushing for 15 min, then removing samples from the suspension for survival tests at different times after addition of the drug.

Diamide was added to the suspensions of aerobic cells 10 to 20 min before irradiation and removed within 15 min after the termination of irradiation. For cells irradiated under extremely hypoxic conditions diamide was added 45 to 60 min before the start of the irradiation and again removed within 15 min after the irradiation was completed. Irradiation and sampling lasted about 20 to 30 min.

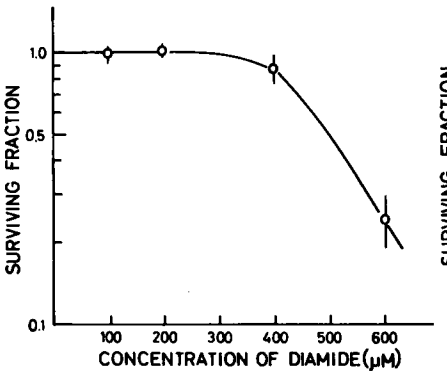

Fig. 1

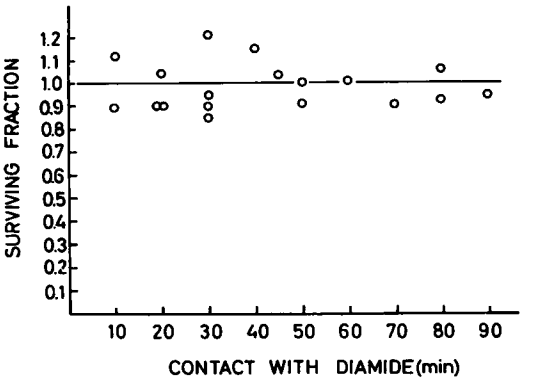

Fig. 2

Fig. 1. The effect of diamide on the reproductive capacity of unirradiated aerobic NHIK 3025 cells in contact with diamide for 2 hours. During this period the cells were suspended in growth medium and held at room temperature. The points represent the mean of 4 independent experiments. The bars represent the standard errors.

Fig. 2. The effect of 200 μM of diamide on the reproductive capacity of NHIK 3025 cells in contact with diamide for different times under extremely hypoxic conditions. The cells were kept under hypoxic conditions even during diamide addition.

All irradiations were performed with 220 kV roentgen radiation. The dose rate was 1.6–1.9 Gy/min (160–190 rad/min) for cells irradiated under extremely hypoxic conditions and 3 Gy/min for cells irradiated under aerobic conditions.

Diamide was obtained from Calbiochem, San Diego, California and used without further purification.

Results

The surviving fraction versus diamide concentration for aerobic cells kept in contact with diamide for 2 hours at room temperature appears in Fig. 1. The symbols represent the mean survival ratios based on 4 independent experiments and standard errors are indicated with bars. The data show 200 μM to be the highest tested concentration without toxic effect under aerobic conditions.

Diamide of a concentration of 200 μM is non-toxic also for cells under extremely hypoxic conditions, for times of at least up to 90 min (Fig. 2). Consequently, in the present experiments diamide was never used in concentrations exceeding 200 μM and the drug was never kept in contact with the cells for more than 90 min.

The effect of diamide of concentration 200 μM on aerobically irradiated cells appears in Fig. 3. Data from 2 different experiments are given. In both experiments duplicate samples were irradiated, e.g. in presence and absence of diamide. No significant modifying effect of diamide under aerobic conditions was found.

The dose-effect curve of extremely hypoxic NHIK 3025 cells irradiated suspended in E2a medium has been published previously (PETTERSEN et coll. 1974b). Now 5

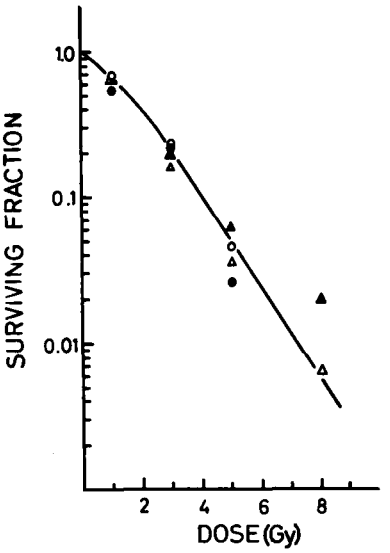

Fig. 3. Dose-effect curve for aerobic NHIK 3025 cells irradiated in presence or absence of diamide at 200 μM. Open symbols represent results for cells irradiated in absence, and closed symbols results for cells irradiated in presence of diamide. Data from two independent experiments are presented, the one represented by triangels and the other by circles.

independent experiments were performed to control the reproducibility of the previous results (Fig. 4). Each type of symbol represents results from one single experiment. The curve is drawn on basis of all points.

The D_0 of the initial exponential line is 5.6 Gy and the D_0 of the exponential line at higher doses (above 18 Gy) is 2.3 Gy, which agrees well with the previous results.

In Fig. 5 data are presented from irradiation experiments with extremely hypoxic cells in contact with diamide of 3 different concentrations. For comparison, the curve from Fig. 4 is redrawn, but without the experimental points. The vertical bars represent the standard errors of the experimental points in Fig. 4.

For diamide concentration of 20 μM no significant sensitizing effect occurred (Fig. 5A). The low protective effect indicated at low doses is not considered significant. This result agrees well with that found by HARRIS & POWER for Chinese hamster cells in presence of 20 μM of diamide and in absence of glucose.

However, for a diamide concentration of 50 μM, the data indicate a low enhancement of radiation inactivation for doses above about 13 Gy (Fig. 5B); for a diamide concentration of 200 μM a clear enhancement for doses above 8 Gy (Fig. 5C). No modifying effect of diamide in the initial dose region (up to 8 Gy) was found for any concentration tested.

Discussion

The toxic effect on NHIK 3025 cells (Fig. 1) differs from that reported for different lines of Chinese hamster cells by HARRIS & POWER. The survival of NHIK 3025 cells drops faster than that of Chinese hamster cells as the diamide concentration ex-

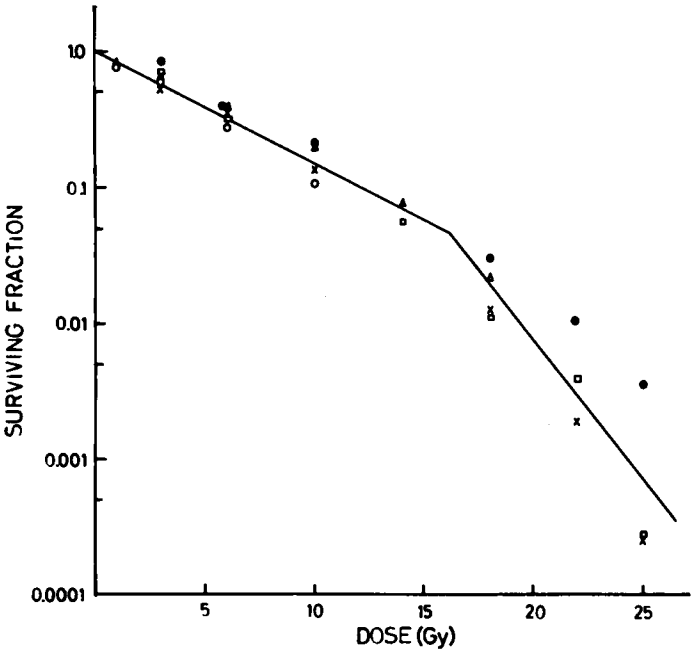

Fig. 4. Dose-effect curve of extremely hypoxic (< 4 ppm O_2) NHIK 3025 cells. Five different and independent experiments are presented, with one type of symbol for each. Exponential lines are drawn by hand.

ceeds 400 μM. However, it is difficult to draw further conclusions from this difference since HARRIS & POWER investigated the toxicity of diamide on cells kept in phosphate buffered saline at 4°C, whereas in the present experiments growth medium at room temperature was used. Furthermore, the time of exposure to diamide was 15 min in the experiments of HARRIS & POWER and 2 hours in the present ones. In spite of these differences the highest non-toxic concentration on aerobic cells is for both groups found to be about 200 μM.

WATTS et coll. have exposed Chinese hamster cells to diamide for 2 hours while the cells were kept in growth medium (MEM + 15 per cent serum), or for 30 minutes while they were kept in Hanks' BBS. In both cases they found a maximum non-toxic concentration of diamide which was lower than 200 μM. When the cells were kept in MEM + 15 per cent serum at 20°C the survival dropped to 75 per cent at 100 μM and to zero at about 300 to 400 μm indicating an even faster drop in survival than was found in the present experiments. This suggests that the degree of toxicity of diamide is dependent on the cell system.

The data in Fig. 3 show that under the present experimental conditions no modifying effect of diamide on aerobic NHIK 3025 cells occurs. This would be considered a prerequisite if the drug were to be used as a sensitizer in radiation therapy. Data published by HARRIS & POWER, CHAPMAN et coll. 1973 b and WATTS et coll.

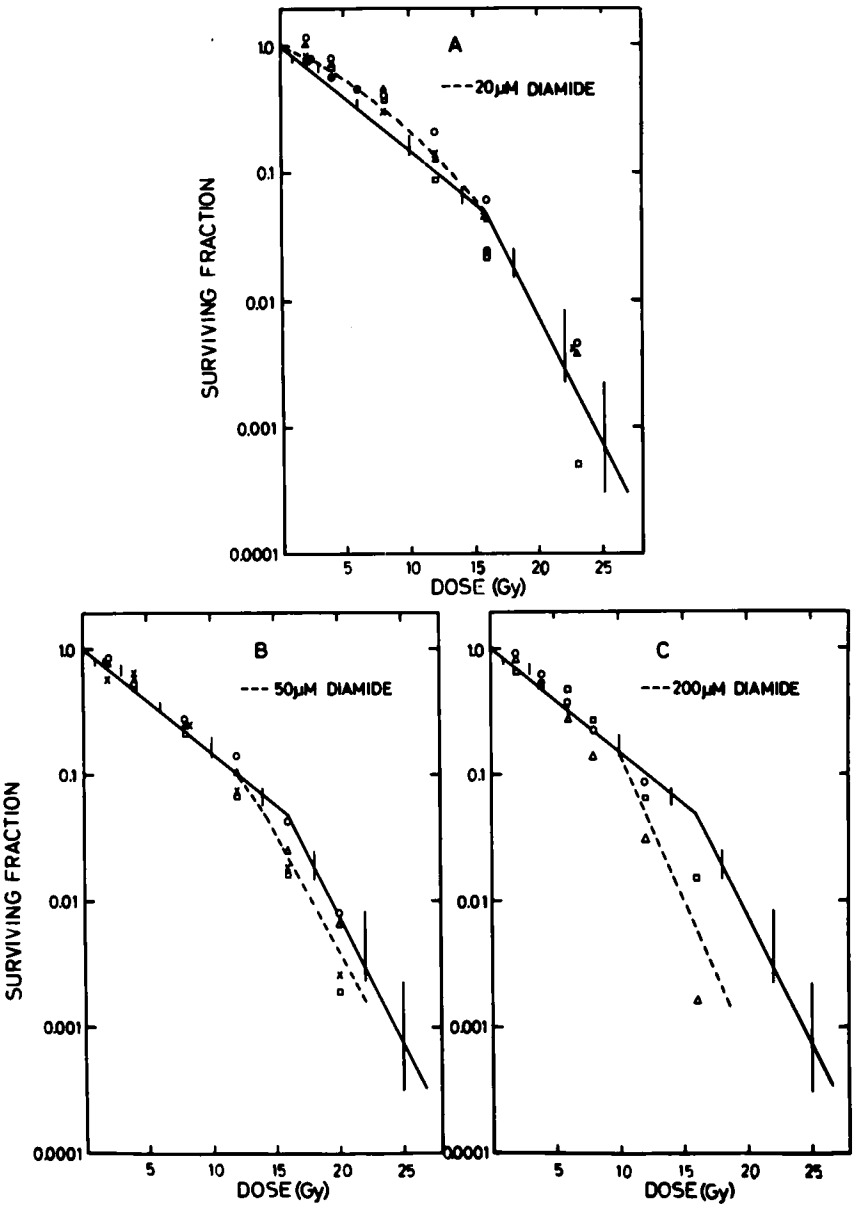

Fig. 5. Dose-effect curves for extremely hypoxic NHIK 3025 cells irradiated in suspension in presence of diamide at 3 different concentrations (20 μM, 50 μM and 200 μM). The dose-effect curve for extremely hypoxic NHIK 3025 cells irradiated in absence of diamide is, to facilitate comparison, redrawn from Fig. 4. The dotted lines are all drawn by hand.

indicate that high concentrations of diamide may have a sensitizing effect on Chinese hamster cells under aerobic conditions. The present results with NHIK 3025 cells irradiated under extremely hypoxic conditions in contact with 50 and 200 μM of diamide, also differ from those published by HARRIS & POWER. They reported that diamide at 50 and 200 μM exerted a strong sensitizing effect at low doses. The present data indicate no sensitizing effect at low doses. On the other hand the present results confirm those of HARRIS & POWER in the sense that diamide does not exert any slope-modifying effect. In their experiments high concentrations of diamide (50 or 200 μM) removed the whole shoulder of the hypoxic survival curve. In the present experiments diamide concentrations of 50 and 200 μM reduced the initial exponential component of the hypoxic survival curve.

WATTS et coll. have published data for the sensitizing effect of diamide on hypoxic cells which have similar trends as the present ones. Hypoxic cells in contact with 100 μM diamide in full medium exhibited no sensitization at low doses ($<$about 4 Gy) but a distinct sensitizing effect appeared at higher doses (i.e. a part of the shoulder had been removed in the presence of diamide). A comparison of the present experimental system with those of HARRIS & POWER and WATTS et coll. suggests that the presence of glucose in the medium eliminates the sensitizing effect of diamide at low doses. This suggestion is supported by the data of HARRIS et coll. (1971) and HARRIS & BIAGLOW which indicate that the ability of diamide to oxidise glutathione is reversed in the presence of glucose.

The results presented by HARRIS et coll. (1974) on hypoxic P388 leucemia cells, grown in vivo as ascites tumours, indicated a dose-modifying sensitization by diamide. In their experiments, the concentration of diamide in the tumours 1 min before irradiation was reported to be approximately 1.7 mM. This concentration is almost 10 times higher than the highest one used in the present experiments. Besides, since it is difficult to estimate the oxygen concentration in the hypoxic tumours, it is correspondingly difficult to compare the present data with the data presented by HARRIS et coll. (1974).

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SUMMARY

Human cells of line NHIK 3025 were irradiated suspended in growth medium (E2a) in absence and presence of diamide under aerobic and extremely hypoxic ($<$4 ppm O_2) conditions. A sensitizing effect of diamide was found for doses exceeding 8 Gy (800 rad) on cells irradiated under extremely hypoxic conditions in presence of diamide of concentration 200 μM, whereas no significant effect was observed for 20 μM.

ZUSAMMENFASSUNG

Humane Zellen der Linie NHIK 3025 wurden in einem Wachstumsmedium (E2a) suspendiert und mit und ohne Diamid unter aeroben und extremen hypoxischen (<4 ppm O_2) Bedingungen bestrahlt. Ein sensibilisierender Effekt von Diamid wurde für Dosen von mehr als 8 Gy (800 rad) bei Zellen unter extrem hypoxischen Bedingungen in Gegenwart von Diamid in einer Konzentration von 200 μM gefunden, während kein signifikanter Effekt für 20 μM zu beobachten war.

RÉSUMÉ

Des cellules humaines de la lignée NHIK 3025 ont été irradiées en suspension dans le milieu de culture (E2a) en l'absence et en la présence de diamide dans des conditions aérobies et dans des conditions extrêmement hypoxiques (<4 ppm O_2). Les auteurs ont constaté un effet sensibilisant du diamide pour des doses dépassant 8 Gy (800 rad) sur des cellules irradiées dans des conditions extrêmement hypoxiques en présence de diamide à la concentration de 200 μM, alors qu'il n'y a pas d'effet observable important pour 20 μM.

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