Volume 249, number 2, 343–347 FEB 07216 June 1989

Induction of tumour hypoxia by a vasoactive agent

A combined NMR and radiobiological study

J.F. Dunn, S. Frostick, G.E. Adams*, I.J. Stratford*, N. Howells*, G. Hogan and G.K. Radda

MRC Biochemical & Clinical Magnetic Resonance Unit, Department of Biochemistry, University of Oxford, Oxford and **MRC Radiobiology Unit, Chilton, Didcot, England*

Received 10 April 1989

The effect of hydralazine treatment on 3 murine tumours (RIF-1, KHT and 16/C) was monitored using ^{31}P-NMR. Changes in the ^{31}P-NMR spectrum are compared with measurements of radiobiological hypoxic fraction (RHF) in the RIF-1 and KHT. Hydralazine is known to reduce temporarily blood flow in experimental tumours, and thus cause a transient increase in the RHF to 100% (in RIF-1 and KHT). This correlates with a decline in energy status as measured by ^{31}P-NMR (i.e. there was an increase in P_i in all three tumours). Time-course data from the RIF-1 and KHT tumours show that maintenance of anaesthesia prolongs the hypoxia induced by hydralazine.

Hydralazine; NMR; Tumor; Chemotherapy

1. INTRODUCTION

Most solid tumours are believed to contain some hypoxic cells, which can make them resistant to radiotherapy [1]. The relationship between oxygen tension and proportion of tumour cells that are hypoxic, the radiobiological hypoxic fraction (RHF), has been studied in detail but how this depends on cellular energetics is not known.

Tumours have been investigated by phosphorus NMR in experimental [2,3] and clinical [4] situations. Metabolic response to therapy [2,5] has been studied and a correlation between blood flow and tumour energy status has been reported [6]. Hydralazine, an anti-hypertensive agent, alters tissue perfusion and increases the resistance of experimental tumours to radiation [7]. This resistance has been attributed to the 'steal' phenomenon, whereby tumour perfusion is decreased when blood flow to other tissues is increased. Induction of severe hypoxia in tumours can greatly increase the effect of bioreductive agents [7,8].

NMR data showing changes in tumour energetics following hydralazine treatment have been reported [9,10], but no attempt has been made to correlate these data with RHF. We now report the results of studies on the effects of hydralazine on three experimental tumours as monitored by ^{31}P-NMR, and in 2 tumours as monitored by changes in the RHF. Two questions addressed were the effectiveness of hydralazine on changing the energy status of these tumours, and whether there is a time-course relationship between the RHF and energy status of tumours.

2. MATERIALS AND METHODS

2.1. *Mice and tumours*

The KHT sarcoma [11], RIF-1 fibrosarcoma [12] and 16/C mammary carcinoma [13] were implanted subcutaneously in category IV CBH/He mice in the mid-dorsal pelvic region of the back. Mice were treated with radiation or subjected to NMR analysis when tumours attained a mean diameter of 6–8 mm.

Correspondence address: J.F. Dunn, MRC Biochemical & Clinical Magnetic Resonance Unit, University of Oxford, Department of Biochemistry, South Parks Road, Oxford OX1 3QU, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2.2. *Tumour irradiation and assay response*

Tumours in mice were locally irradiated with 250 kV X-rays as in [14]. Each tumour was assayed individually [15]. Tumours were excised 24 h after treatment, single-cell suspensions prepared, and appropriate cell members plated as described for KHT [15] and RIF-1 [12] tumours. Following incubation for 14 days, colonies of 50 or more cells were scored as survivors.

2.3. *Animal anaesthesia*

Mice were treated intraperitoneally with a 1:1:2 mixture of hypnorm:hypnovel:H_2O initially at a dose of 0.2 ml per mouse intraperitoneal and then, during the time-course experiments, further doses of 0.1 ml administered at approx. 90 min intervals.

2.4. *NMR spectroscopy*

The experiments were performed at 73.836 MHz using a vertical-bore Oxford Instruments magnet and a Bruker spectrometer. A 0.7 cm diameter double-turn surface coil tuned to ^{31}P and ^{1}H was positioned above the tumour. Magnetic field homogeneity was adjusted whilst observing the proton signal. Spectra were collected as 2048 data points and were the result of 256 transients. The sweep width was 3000 Hz, the recycle time was 3.5 s and the pulse width used corresponded to a maximum signal intensity.

Spectra were transformed using a combination of trapezoidal and exponential multiplication, with a trapezoidal constant of 4 and a line broadening of 20 Hz. Peak areas were obtained by triangulation. Intracellular pH values were calculated from the chemical shift difference between PCr and P_i using the equation $pH = 6.75 + \log(S - 3.27)/5.69 - S)$.

3. RESULTS

3.1. *Radiobiological hypoxic fraction (RHF)*

Hypoxic fractions in tumours are usually determined by in vitro assay of cells irradiated in vivo. Comparison of cellular survival in tumours where the blood supply is temporarily occluded during irradiation by clamping, with data from unclamped tumours allows the estimation of RHF [16]. Following treatment of unanaesthetized mice with hydralazine (5 mg/kg, i.v.) the RHF in RIF-1 and KHT tumours increased to 100% (fig.1) from their normal levels of approx. 1 and 10%, respectively. The surviving cell fractions (SCF) of KHT cells (following 14 Gy irradiation) taken from tumours in which the blood supply was temporarily occluded was 10-fold higher than that of cells from unclamped tumours. The mean SCF (SCF) increased from 0.71×10^2 (0.52–0.91: standard error of the geometric mean) to 6.1×10^2 (5.7–6.5). The value corresponds to the SCF from a completely radiobiologically hypoxic population. An increase in SCF to 6.1×10^2 (5.2–7.1) was also observed in

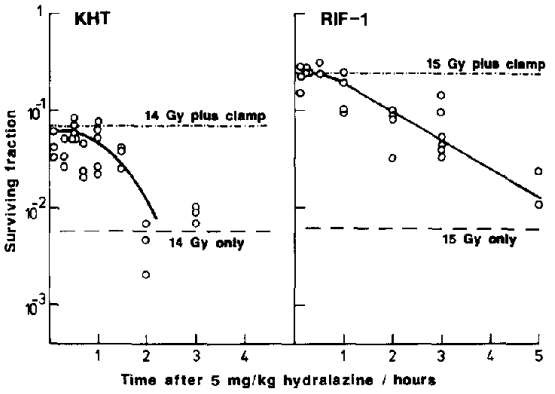

Fig.1. Surviving fraction of KHT and RIF-1 tumour cells at various times after treatment with hydralazine. The lower dashed line gives survival of cells for mice receiving radiation only (i.e. these are control hypoxic fractions of 10% in KHT and 1% in RIF-1). The upper dashed line gives survival of cells from clamped tumours, i.e. the hypoxic fraction is equal to 100%. Curves were fitted by eye.

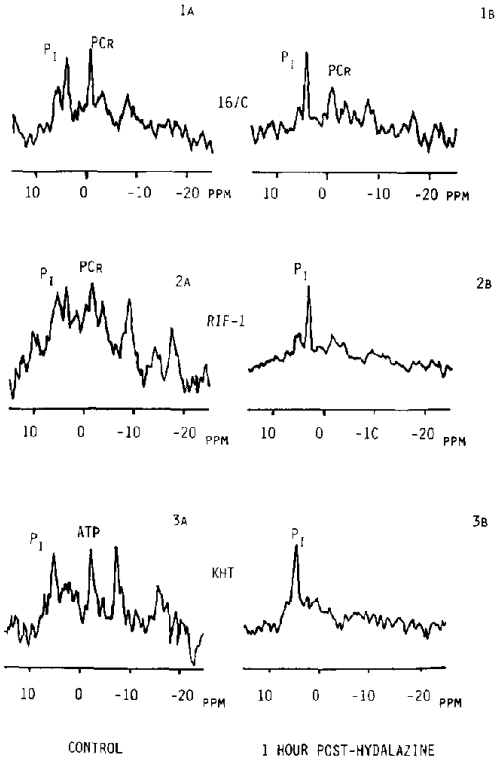

Fig.2. Tumour spectra, control and 1 h post-hydralazine.

tumours from mice treated with hydralazine up to 30 min prior to irradiation. The effect of hydralazine was transient. The SCF returned to normal values at 2–3 h post-hydralazine. Hydralazine also induced 100% radiobiological hypoxia in the RIF-2 tumour except that a longer period was required for the SCF to approximate normal levels (fig.1).

In some experiments, KHT-bearing mice were anaesthetized, injected with hydralazine 1 h later and maintained under anaesthesia for a further 3 h before irradiation. The SCF following this treatment was 6.9×10^2 (4.6–8.5), a value similar to that for the clamped response. This contrasts with the results obtained with hydralazine at 3 h when anaesthetic was not used, i.e. the SCF returned to control values. Anaesthetic alone for 3 h caused a small increase in SCF to 1.8×10^2 (1.2–2.5).

3.2. *NMR*

In one series of experiments, the animals were anaesthetised, placed in the magnet for a control spectrum, removed for hydralazine injection, and then returned to the magnet to follow the time course of the response. Fig.2 shows representative ^{31}P-NMR spectra from untreated tumours (A) and from tumours in mice treated 1 h previously with hydralazine (B). In each tumour examined the spectra showed a decline in high-energy metabolites (PCr and ATP) and an increase in the inorganic (P_i) peak. In many cases, the post-hydralazine spectra consisted solely of the P_i peak. Even after 4 h, there was no observed recovery in the spectra from any of the RIF-1, KHT or 16/C tumours when the mice were maintained under anaesthesia (see fig.3A for RIF-1 and KHT).

In a second series of experiments (on RIF-1 and KHT tumours) the effect of anaesthetic was minimized by giving a single dose of anaesthesia just before collection of the NMR spectra. This was not done on the 16/C tumour as there was no comparative RHF data. Fig.3B shows, for individual tumours, data from various times after hydralazine injection. During the 1.5 h after hydralazine treatment, 4 RIF-1 tumours had only P_i visible in the spectra. Some control phosphorus

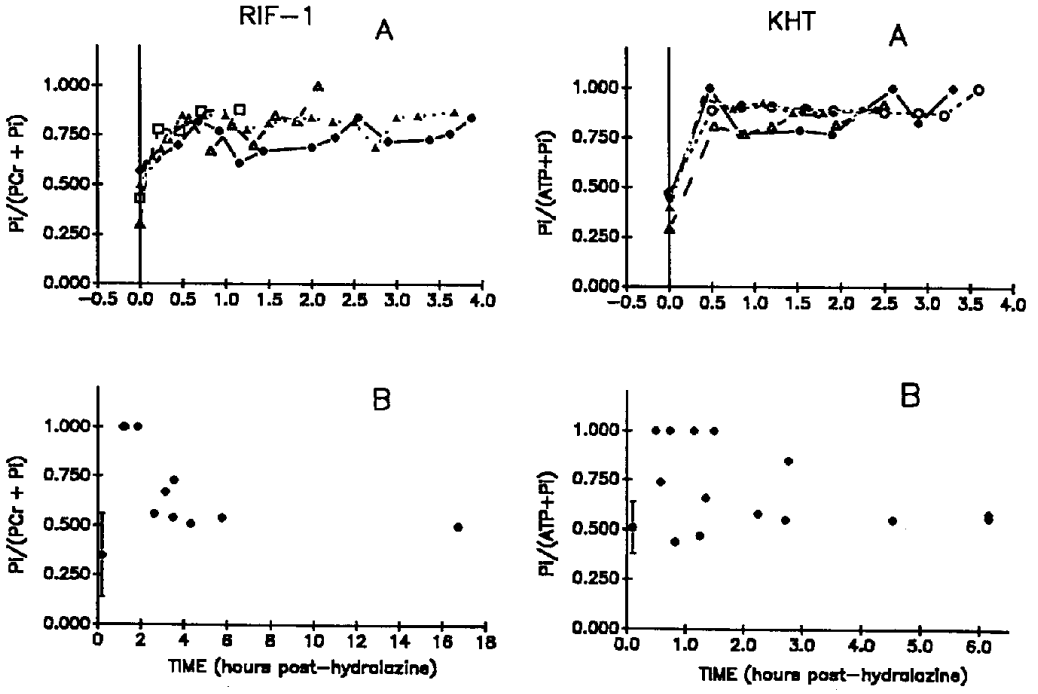

Fig.3. Metabolite ratios from RIF-1 and KHT tumours. (A) Tumour spectra collected serially from the same animal. Each line represents a different animal. (B) Spectra were collected from different animals, each anaesthetized immediately prior to examination. The control point represents $x \pm$ SD (RIF-1, $n = 7$; KHT, $n = 9$).

ratios were approached before 3 h post-treatment, showing that the tumours were recovering over a time scale similar to that for recovery of RHF in unanaesthetised mice. The KHT data, although more scattered, also appears to show an increase in P_i (relative to ATP), followed by some degree of recovery. The more rapid recovery is consistent with the more rapid recovery of a control RHF in KHT tumours.

The control pH values were 7.06 ± 0.15 (KHT, $n = 5$), 7.09 ± 0.18 (RIF-1, $n = 7$), and 7.20 ± 0.20 (16/C, $n = 4$). In the experiments where individual animals were examined, the pH decreased in KHT tumours from the control value to 6.77 ± 0.12 ($p < 0.05$) in animals measured up to 2.8 h post-hydralazine. The mean pH returned to 7.07 ± 0.11 in the post 3 h group. Changes in pH were not measured in the RIF-1 tumour because of the absence of a reference PCr peak immediately following hydralazine treatment.

4. DISCUSSION

The fraction of cells that are radiobiologically hypoxic can be measured by assaying tumour cell survival in vitro following irradiation in vivo. Following treatment with hydralazine, both the RIF-1 and KHT tumours became 100% hypoxic.

The administration of hydralazine also had a marked effect upon the metabolism in the three tumour types, as measured by ^{31}P-NMR. In experiments where the same animal was used for control and post-hydralazine spectra the proportion of P_i relative to ATP and PCr increased and, in KHT, the pH decreased. This is consistent with changes expected on the basis of increased tumour hypoxia. It also indicates that anaerobic glycolysis is unable to provide sufficient ATP for tumour metabolism under these ischemic conditions. From these data one may conclude that, under these conditions, ^{31}P-NMR data can be used to predict changes in RHF. The similar time courses observed when using both NMR and RHF suggest that there is a relationship between hydralazine treatment, oxygen delivery and energy status.

The effect of hydralazine on both the NMR spectra and RHF is dependent upon the duration of anaesthesia. Both studies indicated that during the time course of hydralazine treatment in a non-anaesthetized state, the tumours first became more hypoxic and then showed signs of recovery. In contrast, when hydralazine was administered after anaesthetic and the time course followed with the mouse maintained under anaesthesia, there was no sign of recovery in either tumour after 4 h.

The three tumours used in this study, although of different origin and histological type, are all highly anaplastic transplantable tumours with high growth fraction. Transplantable murine tumours may not be ideal models therefore, for investigating any relationships between the degree of tumour hypoxia and energy status in primary human tumours where the degree of differentiation, growth rate, and other kinetic parameters may be different. It is encouraging however that hydralazine appears to substantially increase hypoxia as measured by RHF in the 2 tumours where RHF can be measured directly and, as measured by ^{31}P-NMR, in all 3 tumours used in this study.

Acknowledgement: This project was funded by the Medical Research Council.

REFERENCES

[1] Bleehen, N.M. (1988) Radiobiology in Radiotherapy, Springer, Berlin.
[2] Evanochko, W.T., Ng, T.C., Lilly, M.B., Lawson, A.J., Corbett, T.H., Durant, J.R. and Glickson, J.D. (1983) Proc. Natl. Acad. Sci. USA 80, 334–338.
[3] Griffiths, J.R., Stevens, A.N., Iles, R.A., Gordon, R.E. and Shaw, D. (1981) Biosci. Rep. 1, 319–325.
[4] Oberhaensli, R.D., Hilton-Jones, D., Bore, P.J., Hands, L.J., Rampling, R.P. and Radda, G.K. (1986) Lancet ii, 8–11.
[5] Le Moyec, L., Naruse, S., Higuchi, T., Hirakawa, K., Watari, H., De Certaines, J.D. and Roques, B.P. (1988) Cancer Biochem. Biophys. 10, 31–45.
[6] Evelhoch, J.L., Sapareto, S.A., Nussbaum, G.H. and Ackerman, J.J.H. (1986) Radiat. Res. 106, 122–131.
[7] Chaplin and Acker (1987) Int. J. Radiat. Oncol. Biol. Phys. 12, 1091.
[8] Stratford, I.J., Godden, J., Howells, N., Embling, P. and Adams, G.E. (1987) in: Radiation Research (Fielden, E.M. et al. eds) vol.2, pp.737–742, Taylor and Francis, London.
[9] Bhujwalla, Z.M., Tozer, G.M., Maxwell, R.J., Field, S.B. and Griffiths, J.R. (1988) Soc. Mag. Reson. Med. Abstr., p.206.
[10] Okunieff, P., Kallinowski, F., Vaupel, P. and Neuringer, L.J. (1988) J. Natl. Cancer Inst. 80, 745–750.
[11] Kallman, R.F., Silini, G. and Van Putten, L.M. (1967) J. Natl. Cancer Inst. 39, 539–549.

[12] Twentyman, P.R., Brown, J.M., Gray, J.W., Franko, A.J., Scoles, M.A. and Kallman, R.F. (1980) J. Natl. Cancer Inst. 64, 595–604.
[13] Cobbett, T.H., Griswold, D.P., Roberts, B.J., Peckham, J.C. and Schabel, F.M. (1978) Cancer Treat. Rep. 62, 1471–1479.
[14] Sheldon, P.W. and Hill, S.A. (1977) Br. J. Cancer 35, 795–808.
[15] Thomson, J.E. and Rauth, A.M. (1974) Radiat. Res. 58, 262–276.
[16] Van Putten, L.M. and Kalman, R.F. (1968) J. Natl. Cancer Inst. 40, 441–451.