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Survival response of a human glioma cell line to hyperthermia associated with rhein

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The effect of association of hyperthermia with the anti-inflammatory drug rhein (RH), 4,5-dihydroxyanthraquinone-2-carboxylic acid, on the clonogenic activity of human glioma cells has been examined. RH inhibits neoplastic growth mainly through an ATP depletion, but thermal cell killing is not mediated by the drug-induced changes in the energy status of the cell. The analysis of the interaction between RH and hyperthermia, performed with the isobolar method, demonstrates an additivity of the response so that the effectiveness of the combined treatment is the result of two independent effects. Although the effect of this combination is purely additive, RH allows us to achieve a pre-established cell killing with exposure times at 42°C, which is generally accepted to be clinically achievable. RH might, therefore, be employed to reduce the side effects of hyperthermia without impairing its therapeutic effectiveness.

Key words: Combined treatments, human glioma cells, hyperthermia, rhein.

Introduction

It is well known that exposure to supranormal temperatures can determine the regression of cancer in animals models and in humans,[1] although the basic mechanisms underlying heat-induced cell death are almost unknown and the clinical therapeutic index remains low.[1,2] In spite of the claimed inherent greater thermosensitivity of neoplastic cells,[3,4] it has been demonstrated that there is very little difference in intrinsic heat sensitivity between transformed cells and their normal counterparts.[1]

The thermosensitivity of mammalian cells depends on several factors such as cell cycle phase, extra- and intracellular pH,[5–7] ability to maintain the energy equilibrium, etc. The capacity to continue energy production by either oxidative or glycolytic metabolism is a fundamental prerequisite for cell survival at elevated temperatures.[8–11] The possibility of maintaining the energy sources clearly depends on the functional integrity of glycolysis and terminal respiration so that it should be possible to enhance the sensitivity of neoplastic cells to hyperthermia through the modulation of their energy metabolism.

Clearly, the modulating agent, in order to be useful for clinical application, should be specific and have a low general toxicity; in this respect, one of the most promising compounds is rhein (RH), an anti-inflammatory drug currently employed in clinical use.[12]

Previous reports from this laboratory have demonstrated that RH affects the energy metabolism of neoplastic cells by inhibiting respiration, and both aerobic and anaerobic glycolysis.[13] The decrease in the oxygen consumption lies in the inhibition of electron transport at the dehydrogenase-coenzyme level,[14] whereas the lower lactate production depends on a reduced glucose uptake.[13] The impairment of the energy-yielding pathways brought about by RH in neoplastic cells results in an inhibition of their biosynthetic processes[15,16] and growth,[17] as well as in a potentiation of the cytotoxic effect of other antiblastic drugs.[18]

Experiments were, therefore, undertaken to examine the potential role of RH in enhancing the cytotoxicity of hyperthermia in the human glioma cell line *in vitro*.

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Materials and Methods

Cells

The cell line used throughout the experiments was established from a surgical specimen of a glioblastoma multiforme.[17] The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2–95% air in RPMI medium (Gibco) supplemented with 2 mM glutamine, antibiotics and 10% fetal calf serum. In the exponential growth phase the plating efficiency was 50–60%.

Drug

RH, kindly supplied by Dr Vittorio Behar, Proter Laboratories, Opera, Milan, Italy, was dissolved in sterile quartz bidistilled water immediately before use. The solution was sterilized by filtration through a 0.22 μm filter (Millex GV, Millipore, Molsheim, France).

Hyperthermic treatment and survival studies

For these experiments 1×10^5 cells were plated in 25 cm^2 culture flasks (Corning). On the fifth day of culture, i.e. during the exponential phase of the growth, an amount of freshly prepared drug solution, to obtain the established concentration, was added to the flasks. Exposure was always 24 h. The hyperthermic treatment was performed by horizontal immersion of sealed 25 cm^2 flasks in a thermostatically controlled, constant temperature, stirred water bath accurate to ± 0.1°C for the established time. Combination studies of RH with hyperthermia were carried out by heating the cells for the established period of time. The medium was then removed and replaced for 24 h with that containing the established RH concentration. To investigate the role of treatment schedule, the cells were exposed to the drug for 24 h before hyperthermic treatment. At the end of incubation the medium was discarded, the cells detached from the plastic surface by 0.02% EDTA for 1 min and counted (Coulter Counter, Model ZM). Known aliquots of cell suspension were dispersed into 80 mm plastic Petri dishes (five dishes for each point) so that colonies would appear after 8–10 days.

Data analysis

The analysis of the interaction of RH and hyperthermia was performed by the isobolar approach, a method which is supposed to offer a general solution to the problem of interaction without invoking mechanisms of action.[10,19–21]

Assay of protein synthetic activity

To evaluate the effect of RH on protein synthetic activity, cultures, during the last hour of exposure to the drug or of hyperthermic treatment, were pulse-labelled with 50 μCi of L-[^{35}S]methionine (specific activity = 1000 Ci/mmol). Monolayers were washed 3 times with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. The cells were detached with EDTA, harvested by low speed centrifugation and lysed by vigorous stirring in a detergent-containing low salt buffer [10 mM Tris–HCl, pH 7.6; 10 mM NaCl; 4 mM $MgCl_2$; 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% NP-40]. Cell lysates were clarified by centrifugation (5000 g, 5 min) and stored at −80°C.

Polyacrylamide gel electrophoresis (SDS–PAGE)

Aliquots of cell lysates corresponding to about 30 000 c.p.m. were mixed with an equal volume of 2 times Laemmli sample buffer and analyzed by one-dimensional electrophoresis on 10% polyacrylamide slab gels according to Laemmli[22]. Runs were carried out for 6 h, at 10 mA/slab. The gels were embedded with DMSO/PPO, dried and exposed to a Kodak-X-ray film, for 3–5 days, at −80°C, with intensifier screens.

Results

Figure 1 shows the effect of 24 h of treatment with RH on the clonogenic activity of human glioma cells. The curve, fitted with the multitarget model, has an initial small shoulder followed by an exponential decrease. The values of D_0 and n, 13.7 and 2.2, respectively, are very similar to those previously reported,[18] thus indicating that no significant modifications in the proliferating activity of this cell line occurred.

The effect of temperature from 40 to 44°C on survival of human glioma cells is shown in Figure 2. The curves, at all temperatures tested, are

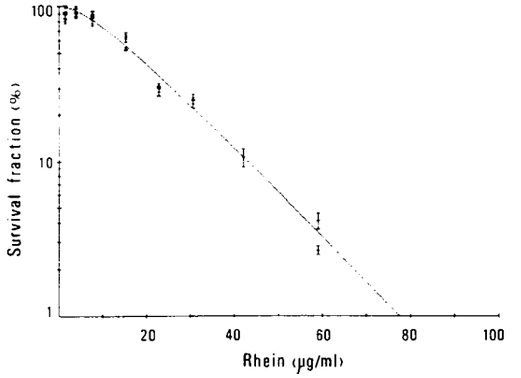

Figure 1. Dose–response curve for exponentially growing cultured human glioma cells exposed to graded doses of RH. The duration of exposure was 24 h. Every line on the vertical bars represents a value of a single experiment. The fitting of the curve was performed with the multitarget model.

characterized by a logarithmic decrease in the survival fraction as a function of the exposure time. The effect of hyperthermia on cell survival is strictly related to the temperature, as demonstrated by the fact that the value of D_0 strongly decreases with the increase of temperature (D_0: 2×10^3 min at 40°C and 32.6 min at 44°C).

To establish the type of interaction, i.e. additive, synergic or antagonist, between RH and heat, the isobologram method was employed.[18,21] The clonogenic activity was evaluated by varying both drug concentration and heating time at 42°C, and by assessing the capacity of each combination to achieve a pre-established cell killing.

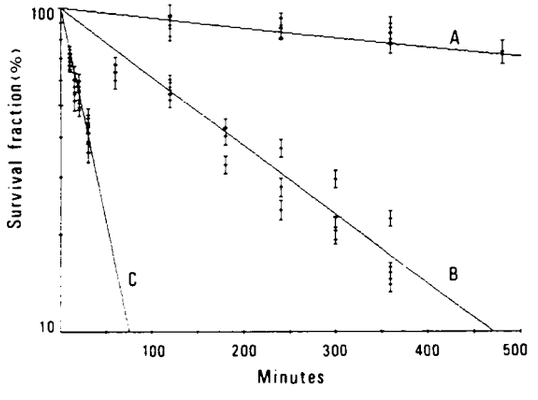

Figure 2. Survival of exponentially growing cultured human glioma cells exposed to **A** 40°, **B** 42 or **C** 44°C for varying periods of time. Every line on the vertical bars represents a value of a single experiment. The fitting was performed with the exponential model.

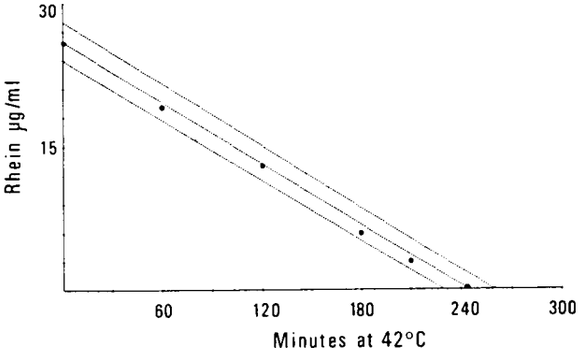

Figure 3. Concentration of RH and heating time at 42°C alone or in combination to obtain 30% survival of exponentially growing cultured human glioma cells. The cells were treated for 24 h with the established RH concentration then followed by the hyperthermic treatment and vice versa. No differences in the response to the two treatment schedules were seen. The upper and lower lines show the zero interaction region. Each combination was repeated at least four times. The error bars are within the closed circles.

Figure 3 shows that 30% survival is obtained either with 25 μg/ml or with 240 min of exposure at 42°C. All combinations tested are clearly additive as demonstrated by the experimental points located in the zero interaction region, or on the straight line joining the concentration of RH and the time of heat exposure that, when given alone, produce the same effect as that in combination.

Furthermore, to verify whether the schedule of treatment might influence the type of response, the cells were previously exposed to the established RH concentration and then to the hyperthermia. As shown in Figure 3, identical results have been observed, thus indicating that neither the extent nor the type of the response are affected by the treatment schedule.

It is well known that in a variety of cells the exposure to supranormal temperature (heat shock) induces or enhances the synthesis of a specific group of heat shock proteins.[23] The synthesis of these proteins is also observed when cells are exposed to other stimuli[24-26] or when there is an imbalance of the energy metabolism.[27,28] In particular, in many cultured cells severe impairment of respiration is one of the early critical biochemical events induced by cell exposure to heat or to other stress stimuli.[29-31]

Because of the ability of RH to interfere with the energy metabolism of the neoplastic cell, experiments were undertaken to ascertain whether the drug was able to modify gene expression. The

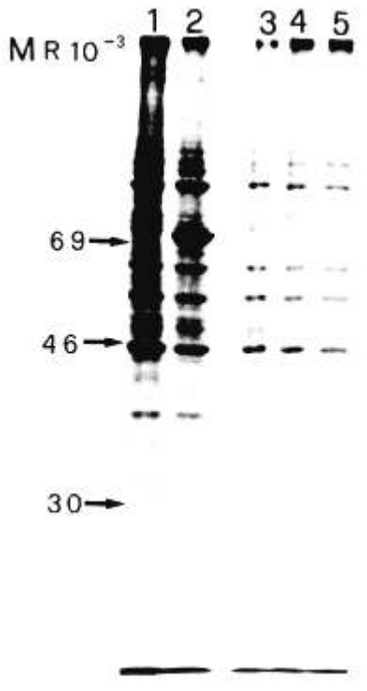

Figure 4. SDS–PAGE analysis of [^{35}S]methionine-labelled polypeptides synthesized in exponentially growing human glioma cells heated at 42°C for 4 h (line 2) or treated with 20 μg/ml RH for 6, 18 and 24 h (lines 3, 4 and 5, respectively). Line 1 refers to control. The respective M_r values are reported on the left side of the figure.

results shown in Figure 4 clearly demonstrate that exposure of the human glioma cells to 42°C for up to 4 h induces the synthesis of a protein band of $M_r = 72\,000$. However, in the cells treated with 20 μg/ml RH the overall protein pattern remains essentially unmodified with only minor modifications in the rate of synthesis of a few polypeptides even if the treatment was extended for 24 h.

Discussion

The data reported here indicate that the effectiveness of the combined treatment does not depend on an interaction between RH and hyperthermia but is the result of two independent effects, as clearly shown by the additivity of the response. In fact, the experimental points of all the combinations tested fall within the zero interaction region with an interaction index very close to 1 (Figure 3).[19]

RH inhibits the growth of neoplastic cells *in vitro*[17,18] but with an effect strictly related to the time of exposure. An exposure time of 4–6 h does not significantly affect the survival[17] that, on the contrary, is strongly reduced by 24 h of treatment (Figure 1). This clearly indicates that the inhibition of neoplastic growth by RH does not depend on impairment of the replicative processes but, on the basis of its mechanism of action,[13,17] is attained mainly through ATP depletion. Therefore, the experimental protocols currently employed in the screening of anti-mitotic drugs may be unsuitable to display the anti-tumor activity of agents which act with different mechanisms, e.g. on energy metabolism.

Human glioma cells are highly dependent on glycolysis for their ATP supply[32] so that RH, by inhibiting glucose uptake[10], as well as oxidative phosphorylation,[14] drastically lowers the adenylate energy charge.[15,16] This reduced ATP availability affects several metabolic processes, such as ion balance, nucleic acids metabolism, and protein and fatty acids synthesis, thus leading to an altered morphological and functional state of the cell. Such alterations can be reversed even though ATP has been low for several hours; only after 18–24 h do these changes become irreversible.[33] Nevertheless, the drug-induced changes in the energy status observed in the present cell line, although drastic,[16] do not appear to mediate thermal cell killing. If so, a lower thermosensitivity should have been observed when drug treatment was performed after hyperthermia.

These results are in agreement with those of Calderwood *et al.*[8] who found that, *in nutritionally adequate media*, the decrease in the adenylate energy charge did not mediate the thermal cell killing which occurs before any significant reduction of the adenylate pool. However, Henle *et al.*[34] found no reduction of the ATP level in Chinese hamster ovary cells after hyperthermic treatment at either 41.5 or 45°C, whereas the cell survival was reduced up to 3 orders of magnitude.

Nevertheless, although in this experimental model the effect of the combination of hyperthermia with RH is purely additive and not synergic, it is worthy of mention that the combined treatments allow us to achieve a pre-established cell killing with exposure times (1–2 h) and with a temperature (42°C) generally accepted to be clinically achievable. If such an effect should also occur *in vivo* the use of this association, considering also the very poor systemic toxicity of RH, might reduce the side effects of hyperthermia without impairing its therapeutic effectiveness.

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References

1. Hanh GM. *Hyperthermia and cancer*. Plenum Press: New York 1980; 233–42.
2. Kim JH, Hahn EW. Clinical and biological studies of localized hyperthermia. *Cancer Res* 1979; **39**: 2258–61.
3. Chen TT, Heidelberger C. Quantitative studies on the malignant transformation of mouse prostate cells by carcinogen hydrocarbons "*in vitro*". *Int J Cancer* 1969; **4**: 166–78.
4. Giovannella BC, Stehlin JS, Morgan AC. Selective lethal effect of supranormal temperatures on human neoplastic cells. *Cancer Res* 1970; **30**: 3944–50.
5. Fellenz MP, Gerweck LE. Influence of extracellular pH on intracellular pH and cell energy status: relationship to hyperthermic sensitivity. *Radiat Res* 1988; **116**: 305–12.
6. Haveman J. The pH of the cytoplasm as an important factor in the survival of *in vitro* cultured malignant cells after hyperthermia. Effect of carbonylcyanide 3-chlorophenylhydrazone. *Eur J Cancer* 1979; **15**: 1281–8.
7. Varnes ME, Glazier KG, Gray C. pH-dependent effect on the ionophore nigericin on response of mammalian cells to radiation and heat treatment. *Radiat Res* 1989; **117**: 282–92.
8. Calderwood SK, Bump EA, Stevenson MA, *et al.* Investigation of adenylate energy charge, phosphorylation potential, and ATP concentration in cells stressed with starvation and heat. *J Cell Physiol* 1985; **124**: 261–8.
9. Gerweck LE, Urano M, Koutcher J, *et al.* Relationship between energy status, hypoxic cell fraction, and hyperthermic sensitivity in a murine fibrosarcoma. *Radiat Res* 1989; **117**: 448–58.
10. Kim SH, Kim JH, Hahn EW. Selective potentiation of hyperthermic killing of hypoxic cells by 5-thio-D-glucose. *Cancer Res* 1978; **38**: 2935–8.
11. Lepock JR, Cheng KH, Al-Qysi H, *et al.* Hyperthermia-induced inhibition of respiration and mitochondrial protein denaturation in CHL cells. *Int J Hyperthermia* 1987; **3**: 123–32.
12. Franchi E, Micheli S, Lovacchi L, *et al.* The influence of RH on biosynthesis of prostglandin-like substances *in vitro*. *J Pharm Pharmacol* 1982; **35**: 262–4.
13. Floridi A, Castiglione S, Bianchi C, *et al.* Effect of RH on the glucose metabolism of Ehrlich ascites tumor cells. *Biochem Pharmacol* 1990; **40**: 217–22.
14. Floridi A, Castiglione S, Bianchi C. Sites of inhibition of mitochrondrial electron transport by rhein. *Biochem Pharmacol* 1989; **38**: 743–51.
15. Castiglione S, Paggi MG, Delpino A, *et al.* Inhibition of protein synthesis in neoplastic cells by rhein. *Biochem Pharmacol* 1990; **40**: 967–73.
16. Delpino A, Paggi, MG, Gentile FP, *et al.* Protein synthetic activity and adenylate energy charge in rhein-treated cultured human glioma cells. *Cancer Biochem Biophys* 1991; **12**: in press.
17. Floridi A, Gentile FP, Bruno T, *et al.* Growth inhibition by rhein and lonidamine of human glioma cells *in vitro*. *Anticancer Res* 1990; **10**: 1633–6.
18. Floridi A, Gentile FP, Bruno T, *et al.* Cytotoxic effect of the association of BCNU with rhein and lonidamine on a human glioma cell line. *Anticancer Res* 1991; **11**: 789–92.
19. Berembaum MC. Criteria for analyzing interaction between biologically active agents. *Adv Cancer Res* 1981; **35**: 269–335.
20. Berembaum MC. The expected effect of a combination of agents: the general solution. *J Theor Biol* 1985; **114**: 413–31.
21. Gentile FP, Chiatti L, Mauro F, *et al.* Interaction of cytotoxic agents: a rule-based system for computer-assisted cell survival analysis. *Anticancer Res* 1992; **12**: 637–44.
22. Laemmli UK. Cleavage of structure of proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 7496–500.
23. Lindquist S. The heat-shock response. *Annu Rev Biochem* 1986; **55**: 1151–91.
24. Johnston D, Oppermann H, Jackson J, *et al.* Induction of four proteins in chick embryo cells by sodium arsenite. *J Biol Chem* 1980; **255**: 6975–80.
25. Levinson W, Oppermann H, Jackson J. Transition series metals and sulphydryl reagents induce the synthesis of four proteins in eukaryotic cells. *Biochem Biophys Acta* 1980; **606**: 170–80.
26. Li GC. Induction of thermotolerance and enhanced heat shock protein synthesis in Chinese hamster fibroblasts by sodium arsenite and by ethanol. *J Cell Physiol* 1983; **115**: 116–22.
27. Haveman J, Hahn GM. The role of energy in hyperthermia-induced mammalian cell inactivation: a study of the effect of glucose starvation and an uncoupler of oxidative phosphorylation. *J Cell Physiol* 1981; **107**: 237–41.
28. Lunec J, Cresswell SR Heat-induced thermotolerance expressed in the energy metabolism of mammalian cells. *Radiat Res* 1983; **93**: 588–97.
29. Delpino A, Nista A, Marcante ML, *et al.* Induction of stress proteins by lonidamine in human and murine melanoma cells. *Exp Mol Pathol* 1986; **44**: 197–206.
30. Floridi A, Nista A, Paggi MG, *et al.* Effect of hyperthermia on electron transport in Ehrlich ascites tumor mitochondria. *Exp Mol Pathol* 1987; **46**: 279–93.
31. Mondovi B, Strom R, Rotilio G, *et al.* The biochemical mechanism of selective heat sensitivity of cancer cell. I. Studies on cellular respiration. *Eur J Cancer* 1969; **5**: 129–36.
32. Paggi MG, Carapella CM, Fanciulli M, *et al.* Effect of lonidamine on human malignant gliomas: Biochemical studies. *J Neuro-Oncol* 1988; **6**: 203–9.
33. Farber E. ATP and cell integrity. *Fedn Proc* 1973; **32**: 1534–39.
34. Henle KJ, Nagle WA, Moss AJ, *et al.* Cellular ATP content of heated chinese hamster ovary cells. *Radiat Res* 1984; **97**: 630–3.

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