Anti-Cancer Drugs, 2, pp. 45–48

1-Hexylcarbamoyl-5-fluorouracil alters the expression of heat shock protein in HeLa cells

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We determined the effect of 1-hexylcarbamoyl-5-fluorouracil (HCFU), a masked compound of 5-fluorouracil, on the expression of heat shock protein (HSP) in heat-treated HeLa cells. We used a monoclonal anti-72-kDa heat shock protein antibody. At 37°C, HSP was predominant in the cytoplasm of cells and heating to 43°C for 30 min increased the synthesis of HSP in the nucleus. When HeLa cells were treated with heat and HCFU, at a concentration which showed evidence of synergy, nuclear staining of the cells decreased. Thus, the altered expression of HSP by HCFU is related to a synergism between heat and HCFU.

Key words: Heat shock protein(s), HeLa cells, 1-hexylcarbamoyl-5-fluorouracil, hyperthermia.

Introduction

The exact mechanism by which heat causes lethality in cells is not well understood. Cellular membranes,[1,2] respiratory metabolism,[3] protein synthesis[4] and DNA[5] are known to be targets for injury by hyperthermia. Despite studies on the efficacy of hyperthermia,[6–11] this modality has no positive effects for some patients. The first step toward elucidation consisted of studies on the induction of thermotolerance, a phenomenon which plays an important role in modifying the cellular response to hyperthermia.

The synthesis of a family of proteins called 'heat shock' proteins is induced or enhanced in cells in response to various environmental stresses. These proteins may perform functions essential to cell survival. Li and Werb reported that heat shock protein (HSP) may play a pivotal role in the development of thermotolerance and would affect transcription in cells.[12] Drugs that show hyperthermic enhancement of cell killing modify the induction of HSP and reduce thermotolerance. We found that 1-hexylcarbamoyl-5-fluorouracil (HCFU), a lipophilic masked compound of 5-fluorouracil (5-FU), used in combination with hyperthermia is more efficacious against HeLa cells than is 5-FU *in vitro*.[13] We report here our observations on modifications of the expression of HSP in HeLa cells exposed to HCFU, using an immunohistochemical method.

Materials and methods

Chemicals

HCFU was obtained from Mitsui Pharmaceutical Inc Ltd (Japan). The drug was dissolved in sterile saline containing 10% dimethylsulfoxide and diluted with medium to produce the desired concentration.

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Cell cultures

HeLa cells were cultured routinely in a monolayer in Eagle's minimal essential medium (Nissui Pharmaceutical Co, Japan) containing 2×10^{-3} M of L-glutamine, 10% fetal calf serum (Gibco Laboratories, Grand Island, USA), 100 units/ml of penicillin, 10 μg/ml of streptomycin and 40 μg/ml of gentamycin. Stock cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Clonogenicity of HeLa cells

For clonogenicity, 3×10^2 cells were seeded into 60 mm plastic dishes without drugs and were maintained for 48 h under the conditions described above. The cells were exposed to 77 μM of HCFU at 43°C for 30 min,[14] then washed three times with phosphate-buffered saline (PBS) and maintained under the conditions described above. The colonies were stained with hematoxylin and counted on day 14, a time when macroscopic colonies were visible, and the fractional cell survival could be computed. The potential of single cells to proliferate and form a colony exceeding 50 cells was assayed.

Immunohistochemical detection of HSP in HeLa cells

For the immunohistochemical detection of HSP, 1×10^5 HeLa cells were grown for 2 days in Lab-Tek tissue culture chamber and slides (Miles Scientific, USA). The slides were washed three times with PBS and exposed to heat (43°C) and/or 77 μM of HCFU for 10–120 min. After washing with PBS, the slides were incubated at 37°C for 3 h. The plastic chambers were then removed and the slides rinsed with PBS. For the immunoperoxidase study we used the avidin–biotin–peroxidase complex method[15] and monoclonal anti-72-kDa HSP antibody (Amersham International plc, UK).[16] Non-specific binding of the second antibody was blocked using normal sheep serum diluted 1:10 in PBS for 20 min at room temperature. Monoclonal anti-72-kDa HSP was applied at the required optimum dilution, in PBS containing 1% bovine serum albumin and 0.1% sodium azide, for 60 min. After washing the slides three times with PBS, biotinylated anti-mouse antibody (Amersham International plc, UK) diluted 1:100 in PBS was applied so as to cover the slides; incubation was then continued for 60 min at room temperature. After washing the slides three times with PBS, streptavidin–biotinylated peroxidase complex (Amersham International plc, UK) was diluted 1:300 in PBS and was applied to the sections. The slides were incubated for 60 min at room temperature and washed three times with PBS. The slides were then covered with diaminobenzidine (DAB) solution, prepared from 0.1% DAB and 0.02% H_2O_2, for approximately 10 min at room temperature and in the absence of direct light.

Evaluation of HSP expression

To evaluate expression of HSPs, its categorization was made as follows. For findings in the nucleus, − indicates no staining, + indicates homogenous staining, and + + indicates staining of nucleoli and a condensed chromatin in the HeLa cells. For findings in the cytoplasm, − indicates no staining and + indicates diffuse staining of the HeLa cells.

Results

HeLa cell clonogenic assay

Colony formation by HeLa cells treated with HCFU at a concentration of 77 μM and heat (43°C, 15–60 min) was inhibited almost completely, in about 1.50% of the control cells after 30 min treatment (Table 1). The combination of HCFU plus heat applied simultaneously led to the greatest cytotoxicity. In contrast, heat alone had little affect on clonogenicity of the HeLa cells. A significant difference in the percentage of colony-forming HeLa cells was noted between HCFU plus heat and HCFU alone ($p < 0.05$) or heat alone ($p < 0.01$). In case of 60 min treatment with heat, clonogenicity was more inhibited and there was no colony formation when heat and HCFU combined were applied.

Table 1. HeLa cell clonogenicity after treatment with HCFU and/or heat (43°C) for 30 min

Treatment	Number of colonies (%)
Heat (43°C)	103.13 ± 9.96
HCFU (77 μM)	38.25 ± 4.53
HCFU + heat	1.50 ± 0.93

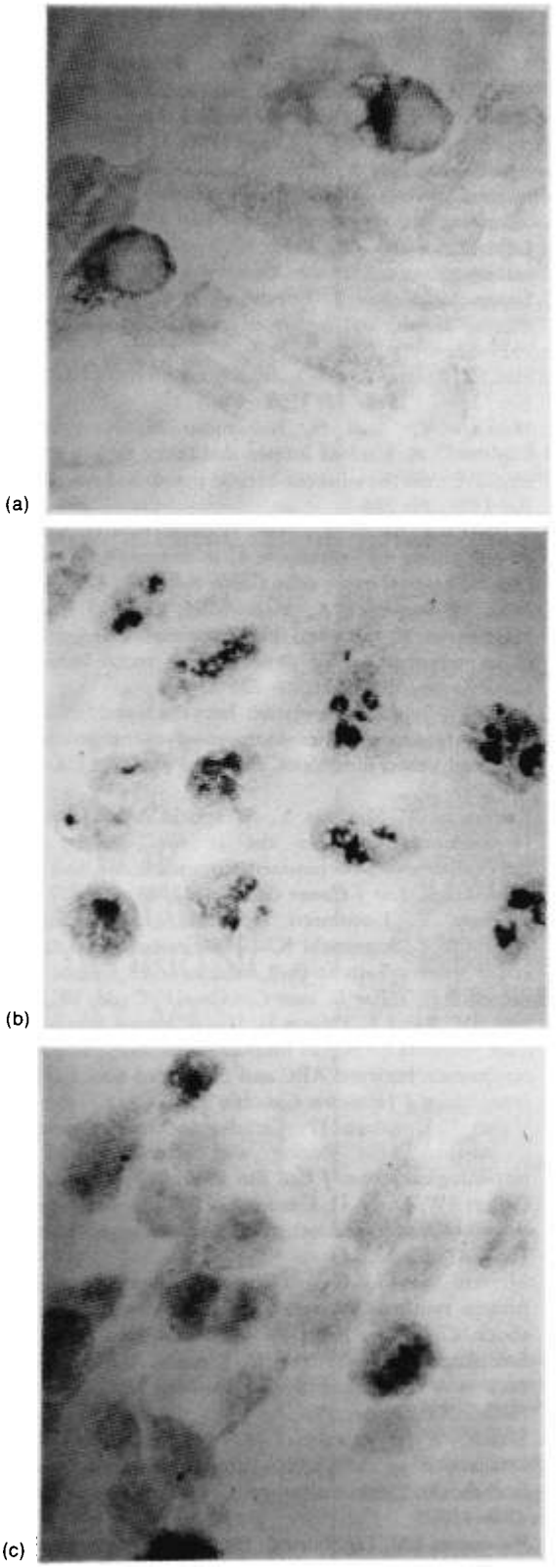

Figure 1. Expression of 72 kDa HSPs in HeLa cells. (a) HeLa cells cultured at 37°C; (b) HeLa cells treated with heat (43°C) for 30 min; (c) HeLa cells treated for 30 min with heat and HCFU combined. Magnifications: ×1020.

Table 2. HSP expression both in the nucleus and in the cytoplasm of HeLa cells after treatment with heat (43°C) and HCFU (77 μM)

Treatment	Expression after the following times (min)				
	0	10	30	60	120
Heat (43°C)	−/+[a]	+/+	++/−	++/−	Unclear
Heat + HCFU	—	+/+	+/+	−/−	Unclear

[a] Findings in the nucleus/in the cytoplasm.

HSP synthesis after treatment with heat (43°C) and HCFU

Diffuse cytoplasmic and some nuclear staining were observed in HeLa cells grown at 37°C (Figure 1a). As early as 10 min following initial heat shock treatment, nuclear staining was more prominent with a slight increase in the cytoplasmic staining compared with findings in unheated controls (Table 2). When HeLa cells were cultured at 43°C for 30 min, the synthesis of HSPs in both the nucleus and the cytoplasm increased, and both granular and patch-like structures appeared (Figure 1b). The nuclear distribution coincided with both nucleoli and/or other areas of chromatin, with varying states of condensation. When the cells were treated with HCFU 77 μM and heat (43°C), the nuclear staining, which had increased after exposure to heat alone, decreased (Figure 1c). The condensed chromatin staining was reduced. After 60 min treatment with heat, the HSPs detected at 30 min treatment were evident, while with the combination there were no such findings. After 120 min treatment with either heat or the combination, the cells were practically destroyed and HSPs were not clearly visible.

Discussion

There is evidence that thermotolerance can be induced in tumors, thereby modifying the cellular response to hyperthermia.[12,17,18] It was reported that thermotolerance can be induced by a short priming heat dose and that it develops rapidly, reaches a maximum within 24 h of the initial treatment, and then slowly decays. Mivechi and Li[18] reported that, when protein synthesis profiles of heat-shocked cells were compared with findings in unheated controls using one- and two-gel electrophoresis, the rates of synthesis of the 70 kDa and 87 kDa proteins were enhanced. The expression of HSPs and the amino acid sequence of several HSPs are highly

conserved throughout evolution,[19] thereby implying that they have an important function, either to protect cells against heat stress or to enhance the potential of cells to recover from the toxic effects of heat and other stress. We found that 72 kDa proteins were synthesized in HeLa cells after a single acute heat treatment at 43°C and that these treatments were sufficient to affect the survival of colony formation of HeLa cells. In addition to the synergistic cytotoxicity between HCFU and heat, we asked whether HCFU would alter the expression of HSP. We had already noted that hyperthermia enhances the activity of both 5-FU and HCFU.[13] Cytotoxicity of HCFU in particular was potentiated more vigorously by heat than by 5-FU. These results led to the notion of possible direct effects of the lipophilic hexylcarbamoyl side chain of HCFU on the cell membrane. Our present observations suggest that HSP synthesis is also altered by the combination HCFU and heat therapy. 72 kDa HSPs were expressed in both the nucleus and the cytoplasm of HeLa cells at temperatures less than 37°C and the expression in the nucleus was enhanced by heat, with HSPs showing a granular and patch-like appearance.[20] HSPs migrate to the nucleus, accumulate there and may act to promote thermotolerance and to preserve configuration of the nucleus.[21] Therefore, considering the observation that HSP expression was inhibited by HCFU, this drug may limit the overall effectiveness of HSPs. HCFU shows promise as an adjunct to heat therapy by limiting the thermotolerance of cells.

Conclusion

In this study, it was suggested that the synergism between HCFU and heat was closely related to the alteration of HSP expression by the drug. As the accumulation of HSPs is the evidence of thermotolerance and preservation of the nucleus, HCFU is capable of adjunct to heat by limiting HSPs.

Acknowledgment

We thank M. Ohara for helpful comments.

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(Received 13 December 1990; accepted 17 December 1990)