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HYDROXYUREA INHIBITS DEGRADATION OF INTERNALIZED EPIDERMAL GROWTH FACTOR IN HELA CELLS

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SUMMARY: Because epidermal growth factor stimulates DNA synthesis in cultured cells, five inhibitors of DNA synthesis were tested in HeLa cells to see whether the inhibition of DNA synthesis has any effect on the metabolism of the growth factor. Among these, only hydroxyurea inhibited the degradation of ^{125}I-labeled epidermal growth factor strongly. The reversal of hydroxyurea-induced inhibition of DNA synthesis by deoxyribonucleosides did not result in a recovery from the inhibition of the degradation. From these findings, it might be concluded that the inhibitory effect of hydroxyurea on the degradation is distinct from that on DNA synthesis.

EGF stimulates DNA synthesis in a variety of cell types (1,2). It has been suggested that the effect of EGF on DNA synthesis might be mediated by a peptide fragment originating from proteolytic cleavage of the internalized hormone-receptor complexes as a second messenger (3). This hypothesis prompted us to test whether the inhibition of DNA synthesis actually affect EGF metabolism, which was extensively investigated using ^{125}I-labeled EGF in a wide variety of cultured cells (1-5). We report here that HU, known as an inhibitor of DNA synthesis (6-8), markedly decreased the rate of degradation of the internalized EGF but revealed no detectable effect on both binding and internalization steps and that no similar effect, however, was observed by the other inhibitors of DNA synthesis. These findings also suggested a new biological activity of HU other than the effect on DNA synthesis.

MATERIALS AND METHODS

DME, calf serum and trypsin were obtained from Gibco. The following materials were from Sigma.: HU, arabinosyl cytocine, cycloheximide, actinomycin D, mitomycin C, 5-fluorodeoxyuridine, dAdo, dGuo, dCyd, bovine

Abbreviations: EGF=epidermal growth factor; DME=Dulbecco's modified Eagle's medium; HU=hydroxyurea; BES=N,N-bis-(2hydroxyethyl)-2-aminoethane sulphonic acid.

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serum albumin and BES. [^{3}H]dThd (20 Ci/mmol), [^{3}H]Urd (40 Ci/mmol) and [^{3}H]Leu (126 Ci/mmol) were from New England Nuclear. Bleomycin A_2 was a gift from Nippon Kayaku Co., Japan. Mouse EGF was from Collaborative Research. Na^{125}I (15.6 mCi/μg I) was from the Radiochemical Center. Tissue culture multiwell plates were purchased from Falcon Labware.

EGF Binding Assay:

HeLa cells were grown in DME plus 10% calf serum in a 5% CO_2-95% air at 37°. For experiment, 1.8 x 10^5 cells per well were inoculated into 17 mm multiwell plates. After 48 hr incubation, confluent monolayers of cells were washed twice with binding buffer consisting of DME containing 1 mg/ml bovine serum albumin and 50 mM BES adjusted to pH 6.8. After a 30 min of preincubation with or without HU, the cells were incubated for the required time intervals at 37° with 0.5 ml binding buffer containing 3 ng of ^{125}I-EGF in the presence or absence of HU. ^{125}I-EGF (specific activity 51863 cpm/ng) was prepared according to the chloramine-T method (9). After incubation, the cells were washed 4 times with cold binding buffer, solubilized with 0.5 ml of 0.5 N NaOH for 1 hr at 37° and cell-associated radioactivity was determined. Nonspecific binding was less than 3% of the total. All data were corrected for nonspecific binding.

Measurement of Down Regulation:

HeLa cells in confluence were incubated at 37° with 6 ng/ml of unlabeled EGF with or without HU. At the indicated times, the cells were washed twice with binding buffer, and 0.5 ml of binding buffer containing 3 ng of ^{125}I-EGF was added per well. The cells were incubated for 1 hr at 37°, and cell-associated radioactivity was measured.

Dissociation of Cell-associated ^{125}I-EGF:

After incubation with ^{125}I-EGF at 6 ng/well for 1 hr at 37°, the cells were washed with binding buffer and 1 ml of fresh binding buffer was added per well with or without HU. The cells were incubated at 37°. At the indicated times, the remaining cell-associated radioactivity was determined.

Incorporation of [^{3}H]dThd, [^{3}H]Urd and [^{3}H]Leu:

The cell were incubated for 4 hr at 37° with 0.5 ml binding buffer containing [^{3}H]dThd (1 μCi/ml), [^{3}H]Urd (1.5 μCi/ml) or [^{3}H]Leu (3 μCi/ml) and various agents. After the incubation, the cells were washed and removed with 0.25% trypsin. The average viable cell number per well was 5.3 x 10^5 cells. After the centrifugation at 3000 rpm for 10 min, acid-insoluble radioactivity was measured.

Effect of Various Agents on Degradation of ^{125}I-EGF:

HeLa cells in confluent monolayer were incubated with ^{125}I-EGF at 3 ng/well for 1 hr at 37°. The cells were subsequently washed 4 times with binding buffer. The cell-associated radioactivity present at this time was taken as the initial zero time control value. One ml of binding buffer containing the various test agents was then added. The cells were incubated for 4 hr at 37°, washed 4 times with binding buffer and cell-associated radioactivity was measured.

RESULTS

The time course of ^{125}I-EGF binding to HeLa cells in the presence or absence of HU are given in Fig.1a. The initial binding of ^{125}I-EGF measured at 1 hr was not affected by 20 mM HU. However, continued incubation up to 6 hr with HU led to an increase in cell-associated radioactivity. Fig.1b shows the effect of HU concentration on ^{125}I-EGF binding to HeLa cells after incubation for 1 or 6 hr. The initial binding was not affected by the concentrations below 20 mM of HU although it was suppressed by 80 to 60% at higher concentra-

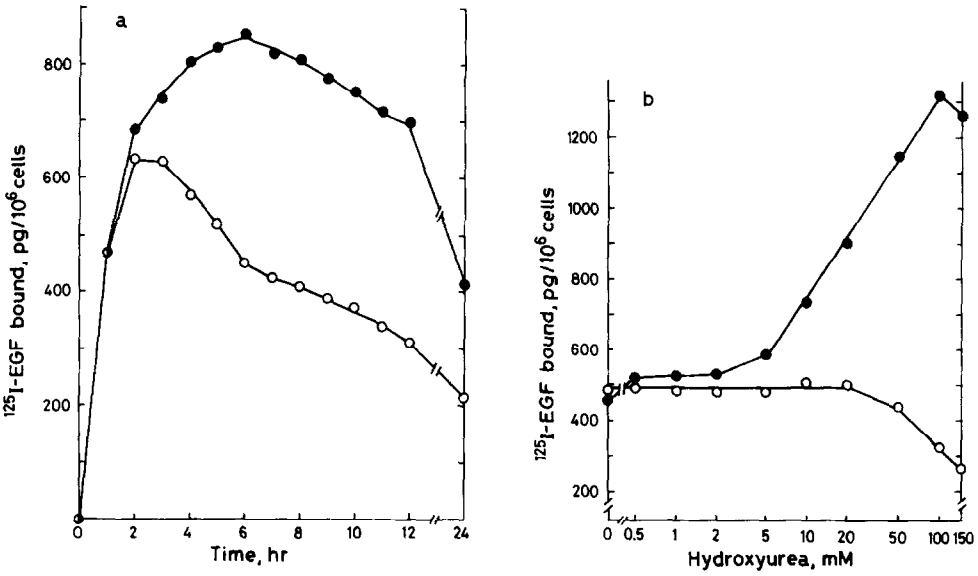

Fig.1 Effect of HU on ^{125}I-EGF binding to HeLa cells. After 30 min preincubation at 37° with or without HU, cells were incubated for the indicated periods at 37° with 0.5 ml binding buffer containing 3 ng of ^{125}I-EGF in the presence or absence of HU. Cells were washed and solubilized for the measurement of radioactivity. Each point represents the mean of three determinations.

a) Time course of ^{125}I-EGF binding to HeLa cells in the absence (○) or presence (●) of HU (20 mM).
b) Effect of HU concentration on ^{125}I-EGF binding to HeLa cells. Cell-associated radioactivity was determined after 1 hr (○) or 6 hr (●) of incubation at 37° with ^{125}I-EGF in the presence of various concentrations of HU.

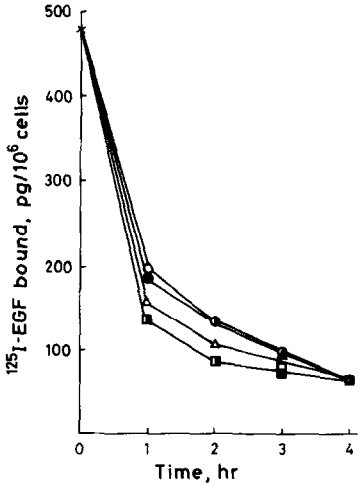

Fig.2 Down regulation of EGF receptors by EGF in the absence or presence of HU. The cells were incubated at 37° with 6 ng/ml of unlabeled EGF in the presence or absence of HU. At the indicated times, cells were washed and 0.5 ml of binding buffer containing 3 ng of ^{125}I-EGF was added per well. After 1 hr of incubation, cell-associated radioactivity was measured. Each point represents the mean of duplicate determinations.
HU concentrations are: ○,none; ●,20 mM; △,50 mM; ■,100 mM; □,150 mM.

tions of 50 to 100 mM. After 6 hr binding period, the amount of radioactivity increased with doses of HU from 5 up to 100 mM. Thus, the increase in cell-associated ^{125}I-EGF induced by HU was not due to an increase in the binding of ^{125}I-EGF to cell surface receptors. Fig.2 shows down regulation (10) of EGF receptors by EGF. After 1 hr of preincubation with unlabeled EGF at 6 ng/ml in the presence or absence of HU, the binding of ^{125}I-EGF was 30 to 40% of the value obtained from no preincubation control and by subsequent incubation, the cell-associated radioactivity decreased in a similar manner regardless of the presence or absence of HU. These findings suggest that HU dose not inhibit the internalization step.

Fig.3 shows the time course of dissociation of cell-associated ^{125}I-EGF in the absence and presence of HU. In the absence, the radioactivity decreased rapidly with a half life of about 50 min. In the presence, however, apparent half life was about 7 hr. When HU was removed from the culture wells at 4 hr of incubation period, the radioactivity was rapidly decreased at a similar rate as observed without HU, indicating the reversible nature of this HU effect.

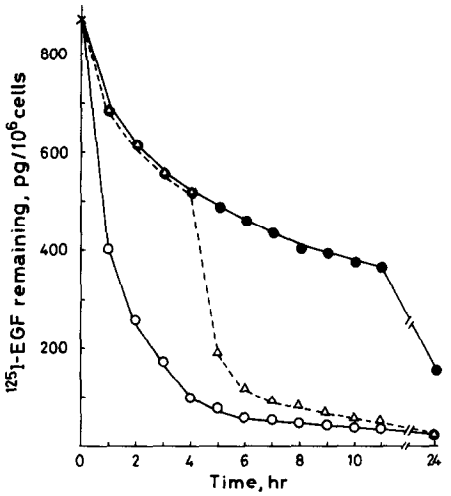

Fig.3 Dissociation of cell-associated ^{125}I-EGF. After incubation with ^{125}I-EGF at 6 ng/well for 1 hr at 37°, cells were washed and 1 ml of fresh binding buffer was added per well with (●) or without (○) 50 mM of HU. At the indicated times, binding buffer was removed and remaining radioactivity was determined. (△): After incubation for 4 hr at 37° with HU (50 mM), binding buffer containing HU was replaced by fresh binding buffer without HU. After further incubation for the indicated time intervals, the cell-associated radioactivity was measured.
Each point represents the mean of duplicate determinations.

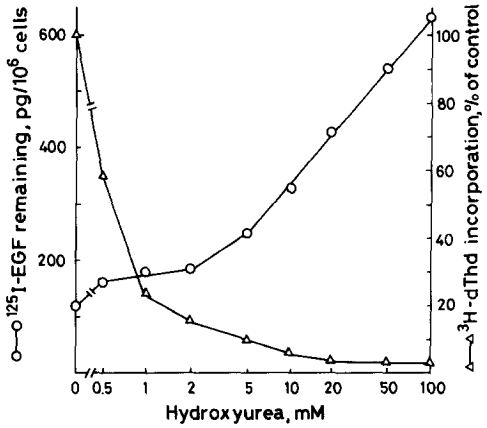

Fig.4 Effect of HU concentration on dissociation of cell-associated ^{125}I-EGF and [^{3}H]dThd incorporation. Cells were preincubated with ^{125}I-EGF and washed as described in Fig.3. After incubation for 4 hr at 37° in 1 ml of binding buffer containing the various concentrations of HU, cell associated-radioactivity was measured (○). In a separate group of assays, cells were incubated for 4 hr at 37° with 0.5 ml binding buffer containing [^{3}H]dThd (1 μCi/ml) and the indicated concentrations of HU (△). All the values were the mean of triplicate determinations.

Dose response curve of HU on the degradation of ^{125}I-EGF and [^{3}H]dThd incorporation into the cells were depicted in Fig.4. The treatment with greater than 2 mM of HU resulted in an increase of the remaining cell-associated radioactivity with doses of the drug up to 100 mM. It should be noted that HU concentrations which inhibited DNA synthesis by about 80% affected ^{125}I behavior only slightly. This implies that the effect of HU to inhibit EGF degradation might be unrelated with its inhibitory action for DNA synthesis.

Therefore, we further investigated to determine whether the effect of HU on inhibition of degradation was actually coupled with the inhibition of DNA synthesis (Table I). [^{3}H]dThd incorporation into HeLa cell DNA was 7.9% of control in the presence of 20 mM HU alone and was recovered to 59.4% by the addition of three deoxyribonucleosides (7,11). Although equivalent inhibition of dThd incorporation was obtained with HU alone at a concentration of about 0.5 mM, no effect on the degradation of ^{125}I-EGF was observed. On the other hand, the effect of HU observed at higher concentrations (20, 50 or 100 mM) on the degradation of ^{125}I-EGF was fully maintained even in the presence of three deoxyribonucleosides under which conditions [^{3}H]dThd incorporation was greatly recovered. Thus, the reversal of HU-induced inhibition of DNA synthesis did

Table I. Effect of Various Agents on DNA, RNA and Protein Synthesis and Degradation of ^{125}I-EGF

agents	[^{3}H]dThd incorp.*	[^{3}H]Urd incorp.*	[^{3}H]Leu incorp.*	^{125}I-EGF remaining**
		% of control		% of initial value
none	100	100	100	14.9
hydroxyurea				
0.5 mM	58.0			15.0
1.0 mM	23.2			15.9
1.5 mM	16.0			16.6
20.0 mM	7.9	98.7	12.1	37.1
50.0 mM	7.6	89.4	10.8	51.3
100.0 mM	5.8	64.4	9.2	57.8
20 mM+3NdR^{+}	59.4			37.1
50 mM+3NdR^{+}	29.4			52.4
100 mM+3NdR^{+}	27.5			57.6
3NdR^{+}	86.4			16.3
5-fluorodeoxy uridine				
0.01-50 mM	90.5-3.1			16.0-13.7
arabinosyl cytocine				
10^{-2}-10^{-6} M	22.1-3.0			14.7-15.4
mitomycin C				
10^{-6}-5x10^{-4} M	98.2-15.0			15.1-13.1
bleomycin A2				
10^{-6}-10^{-3} M	91.6-68.5			14.5-12.6
actinomycin D				
10 μg/ml		6.4		14.6
cycloheximide				
200 μg/ml			8.1	14.6

* Control values of incorporation of [^{3}H]dThd, [^{3}H]Urd and [^{3}H]Leu were 52697 ± 1609, 56843 ± 1900 and 66019 ± 804 cpm/10^{6}cells respectively.
** The initial zero time radioactivity was 45121 ± 2530 cpm/10^{6} cells.
\+ 3NdR represent the mixture of dAdo (0.1 mM), dGuo (0.1 mM) and dCyd (1 μM).
All the values were the mean of three determinations.

not produce alteration of effect of HU on degradation of ^{125}I-EGF. Other inhibitors of DNA synthesis, arabinosyl cytocine, 5-fluorodeoxyuridine, mitomycin C and bleomycin A_2, did not exhibit any significant effect on degradation process of ^{125}I-EGF at the various concentrations tested. These findings highly suggested that the inhibitory effect of HU on the degradation of EGF might be independent from that on DNA synthesis. HU was also shown to inhibit the incorporation of [^{3}H]Urd and [^{3}H]Leu into HeLa cells at high concentrations. The direct correlation between these activities and the effect on EGF degradation was less probable, since both actinomycin D and cycloheximide revealed no effect.

DISCUSSION

From this study it was suggested that HU inhibited degradation process of internalized EGF. This effect was unexpected and was so far undiscovered. The degradation of EGF was reported to be suppressed by several kinds of protease inhibitors, leupeptin and antipain (12), or tosyl-L-lysine chloromethyl ketone and benzyl ester of guanidobenzoic acid (4,9) probably by inhibiting the lysosomal proteases. In our preliminary experiment (unpublished), however, no effect of HU on lysosomal cathepsin B was observed both in vivo and in vitro assays. It is also unlikely that HU inhibits the production of metabolic energy required for the degradation of EGF, since a number of inhibitors of metabolic energy production, such as dinitrophenol, sodium azide or sodium cyanide had little influence on the degradation of EGF in the standard medium containing both glucose and amino acid (9).

Antitubulin agents, such as colchicine, vinblastine, colcemid, and podophyllotoxin, are also known to inhibit EGF degradation (13). It seems, however, unlikely that HU causes depolymerization of microtubules giving rise to an inhibition of the fusion of endocytic vesicles with lysosomes.

HU-induced inhibition of EGF degradation might be caused by a direct effect on lysosomal function in a similar manner that occurs with some kinds of agents such as ammonium chloride (14), chloroquine (15,16) or some tertiary amines (9) and other lysomotropic amines (17).

We are now further investigating the effect of HU on the lysosomal enzyme activity to make these ambiguity clear. Whatever the mechanism would turn out to be, HU should become a useful agent for study of internalized but degradation inhibited EGF, since the major part of the action of HU as an inhibitor of DNA synthesis can be reversed by deoxyribonucleosides.

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REFERENCES

1. Carpenter,G., and Cohen,S.(1979) Annu.Rev.Biochem.48,193-216.
2. Aharonov,A.,Pruss,R.M., and Herschman,H.R.(1978) J.Biol.Chem.253,3970-3977.
3. Das,M., and Fox,F.(1978) Proc.Natl.Acad.Sci.USA.75,2644-2648.
4. Carpenter,G.,Lembach,K.J.,Morrison,M.M., and Cohen,S.(1975) J.Biol.Chem. 250, 4297-4304.
5. Gorden,P.,Carpentier,J.L.,Cohen,S., and Orci,L.(1978) Proc.Natl.Acad.Sci. USA.75,5025-5029.
6. Young,C.W., and Hodas,S.(1964) Science 146,1172-1174.
7. Adams.R.L.P., and Lindsay,J.G.(1967) J.Biol.Chem.242,1314-1317.
8. Masuda,H., and Endo,H.(1981) J.Natl. Cancer Inst.67,1063-1070.
9. Carpenter,G., and Cohen,S.(1976) J.Cell Biol.71,159-171.
10. Raff,M.(1976) Nature 259,265-266.
11. Young,C.W.,Schochetman,G., and Karnofsky,D.A.(1967) Cancer Res.29,291-295.
12. Savion,N.,Vlodavsky,I., and Gospodarowicz,D.(1980) Proc.Natl.Acad.Sci.USA. 77,1466-1470.
13. Brown,K.D.,Friedkin,M., and Rozengurt,E.(1980) Proc.Natl.Acad.Sci.USA.77, 480-484.
14. Seglen,P.O.(1975) Biochem.Biophys.Res.Commun.66,44-52.
15. Wibo,M., and Poole,B.(1974) J.Cell.Biol.63,430-440.
16. Lie,S.O., and Schofield,B.(1973) Biochem.Pharmacol.22,3109-3114.
17. King,A.C.,Hernaez-Davis,L., and Cuatrecasas,P.(1980) Proc.Natl.Acad.Sci. USA.77,3283-3287.