EFFECTS OF PH ON CYTOTOXICITY OF CARBOQUONE

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Abstract—To assess the effects of pH on the cytotoxicity of carboquone (CQ), use was made of the mouse tail skin and HeLa cells in culture. CQ had the most potent cytotoxic effect at pH 6 rather than at pH 7 or 8. Regarding the interaction between 14C-CQ and HeLa cells, both the intracellular accumulation of free 14C-CQ and the ratio of bound 14C-CQ to total 14C-CQ uptake were enhanced at pH 6. Among the fractionated biomolecules of DNA, RNA and protein, DNA was the most active in binding CQ, under the same conditions of pH. The 14C-CQ binding to nucleic acids at pH 6 was more apparent than was the binding to protein. Thus, the enhancement of CQ cytotoxicity at low pHs is probably due to an increase in the intracellular accumulation of free CQ as well as to an enhanced reactivity of CQ with DNA, within the ranges of a lower pH.

One of the characteristics of cancerous tissues is a low pH, possibly due to an accumulation of lactic acid following active glucose uptake and potent glycolysis by the cancer cells (1, 2). This pH difference between cancerous and normal tissues is even more evident when glucose is administered to the host (3).

We carried out a series of experiments in vitro to screen effective anti-cancer drugs, under the acidic conditions induced by tumor glycolysis (4). Among the drugs tested, carboquone (CQ) was found to have the most potent cytoidal effect, particularly with concomitant administrations of glucose.

Using the system of blood flow-interrupting hyperthermic chemotherapy, we have found that the anticancer effect of CQ was notably enhanced when tumor-bearing mice were pretreated with glucose (5). Since these results suggested that CQ had a greater cytotoxic effect under acidic conditions, studies were done in an attempt to elucidate the mode of cytotoxic action of CQ, using experimental systems of a simplified design.

MATERIALS AND METHODS

Chemicals: One mg of carboquone (CQ) was dissolved in 100 mg of N,N-dimethylacetamide and 0.5 ml of polyethylene glycol-400 as the solvents. The solution was used as an original stock and was appropriately diluted with physiological saline just prior to each experiment. There was no evidence of cytotoxic effects of these solvents at the doses used. 14C-CQ (28.97 μCi/mg) was a gift from the Sankyo Co. Ltd; Aquasol (Scintillation solution) was purchased from New England Nuclear Co.

Skin test: Three to five month-old ddN mice of both sexes were obtained from the Animal Center of Kyushu University. Fifty μl of 0.2 M phosphate buffer at pH 6, 7 and 8 containing CQ at appropriate concen-
trations was given subcutaneously into the tail. The localized lesion was observed 5 to 10 days after this treatment. The degree of redness, swelling, depilation, erosion and atrophy was expressed as −, +, and ++.

Assay of cytotoxicity of CQ to HeLa cells: HeLa cells were cultured in Eagle’s Minimum Essential Medium supplemented with 10% fetal calf serum (10% FCS-MEM: pH 7.2–7.4), in a humidified atmosphere of 5% CO₂–95% air at 37°C. In the assay, HeLa cells (250 to 50,000 cells per plate) were placed in 50 mm Falcon plastic dishes and cultured for 6 hr. After removal of the medium, 2 ml of 10% FCS-MEM at pH 6, 7, and 8 (adjusted with 1 N NaOH or 1 N HCl) containing CQ was added to each preparation and incubations were carried out at 37°C for 30 min in “room air” (to avoid pH change of media). At the end of incubation, the medium was poured off, and the cells were washed with 2 ml physiological saline. To the treated cells was added 2 ml of fresh 10% FCS-MEM (pH 7.4) and the cells were re-cultured. On day 8, the number of colonies was counted following the staining with methylene blue. Survival rate was calculated as follows: (the number of colonies of cells treated under various conditions)/ (the number of colonies of the cells treated with medium at pH 7 free of CQ).

Interaction of ¹⁴C-CQ with HeLa cells: HeLa cells in an exponential growth phase were used in labelling experiments. 5×10⁵ HeLa cells in a Petri dish were incubated in 10% FCS-MEM adjusted to each pH (6, 7, and 8) containing ¹⁴C-CQ (5 μg/ml, 28.97 μCi/mg) at 37°C for 30 min in room air. At the end of incubation the reaction media were removed, and the cells were washed twice with 2 ml of cold physiological saline. To estimate total uptake of ¹⁴C-CQ by the cells, 2 ml of 0.2 N NaOH was added to the washed cells, followed by incubation for 60 min at 37°C to lyse the cells. To a 1.5 ml sample was added 10 ml of Aquasol for the measurement of radioactivity. Another sample of 0.2 ml was used for protein estimation by the method of Lowry et al. (6).

For estimation of the amount of ¹⁴C-CQ bound to the cells, 2 ml of cold 95% ethyl alcohol was added after washing the cells with physiological saline, followed by incubation for 1 hr at 4°C to extract unbound ¹⁴C-CQ. The extract was removed and the cells were washed with 2 ml of cold 95% ethyl alcohol and then 2 ml of 0.2 N NaOH was added. Free (unbound) ¹⁴C-CQ was calculated by subtracting the amount of ¹⁴C-CQ from that of total ¹⁴C-CQ.

Estimation of ¹⁴C-CQ bound to RNA, DNA, and protein in HeLa cells: After removing free ¹⁴C-CQ with 95% ethyl alcohol at 4°C, the extraction of RNA and DNA was carried out according to a modified method of Ogur and Rosen (7). Protein was extracted from the final residue with 1 N ammonia at 50°C for 30 min.

The concentrations of nucleic acids (DNA and RNA) and protein of the extracts were determined from the absorbance at 260 nm and 280 nm, respectively, using a Hitachi spectrophotometer.

Ten ml of Aquasol was added to the extracts and the radioactivity was counted using a Beckman liquid scintillation spectrometer. The counting efficiency for ¹⁴C-CQ was not affected by any of the solvents used.

RESULTS

Skin test: As a simple in vivo experiment, the skin test on the tail of mice was used. The findings in the localized lesions following application of CQ at pH 6, 7, and 8 are shown in Table 1. CQ produced severe, local lesions under conditions of pH 6. Although other anticancer drugs, chromomycin A₃, thio-TEPA, mitomycin C, and nitrogen mustard-N-oxide were also tested (data not shown), the enhancement of action at pH
Table 1. Tail skin test in mice

| Drug          | pH | Redness | Swelling | Depilation | Erosion | Atrophy |
|---------------|----|---------|----------|------------|---------|---------|
| Carboquone    | 6  | ++      | ++       | ++         | ++      | ++      |
| (0.1 mg/ml)   | 7  | +       | ±         | ±          | -       | ±       |
|               | 8  | ±       | -         | -          | -       | -       |

Mouse tail skin test for pH dependency of CQ. 50 µl of CQ (0.1 mg/ml) was given s.c. into the tail of mice (ddN ~) and the localized lesions were observed 8 days later.

6 was most evident only in the case of CQ. Administration of 50 µl of 0.2 M phosphate buffer of each pH produced no such localized lesions.

**Cytotoxicity of CQ to HeLa cells:** The survival curve of HeLa cells treated with CQ at 37°C for 30 min is shown in Fig. 1. The degree of effectiveness of CQ was significantly increased at pH 6 as compared with pH 7 and 8. The doses of 90% cell killing were 0.03 µg/ml at pH 6, 0.045 µg/ml at pH 7 and 0.065 µg/ml at pH 8.

The survival of HeLa cells, when incubated with media at various pHs, was not affected by change in the pH alone under conditions of the present experiments.

**Binding of 14C-CQ to HeLa cells:** The total uptake of 14C-CQ by the cells and binding to macromolecules in the cells showed a tendency toward increase when the pH in media was lowered (Fig. 2a). Both the ratio of bound 14C-CQ to total 14C-CQ uptake, (which indicates the degree of the interaction of CQ with macromolecules), and the amount of free 14C-CQ during a 30 min incubation period were greater at pH 6 (Fig. 2b and 2c).

**Binding of 14C-CQ to intracellular RNA, DNA and protein:** 14C-CQ binding to DNA was remarkably higher than either RNA or protein at all pHs examined, and the most evident enhancement was at pH 6. Though 14C-CQ binding to RNA was lower than DNA, an enhancement of 14C-CQ binding to RNA at pH 6 occurred to the same extent, as in the case of DNA. The bound 14C-CQ in protein was also lower than in the case of DNA. However, the amount of 14C-CQ bound to protein was much the same level at all pHs tested (Fig. 3).

In other experiments, the direct interaction of 14C-CQ with DNA (calf thymus DNA) was examined in vitro in phosphate buffer at various pHs (data, not shown). The amount of bound 14C-CQ to DNA was also larger under the in vitro acidic conditions.

**DISCUSSION**

We have confirmed that the cytotoxicity of CQ in mouse tail skin and HeLa cells in culture is enhanced under conditions of pH 6 but not pH 7 and 8. Similar results were obtained with E. coli and S. typhimurium (S.T., in preparation). Therefore, conditions
Fig. 2. pH dependency of reaction of $^{14}$C-CQ with HeLa cells. (a) Amount (○: pH 6, △: pH 7, □: pH 8) of $^{14}$C-CQ uptake by the cells and that (●: pH 6, ▼: pH 7, ■: pH 8) of $^{14}$C-CQ bound to the cells. Each point (with a bar) shows the average of three determinations ± S.E.. (b) Binding ratio of $^{14}$C-CQ to the cells, which was calculated by the following formula.

$$\text{Binding ratio} = \frac{\text{average amount of bound }^{14}\text{-C-CQ}}{\text{average amount of total }^{14}\text{-C-CQ uptake}}$$

Bars: S.E. (c) Increase of intracellular free $^{14}$C-CQ for 30 min. Bars: S.E.

Fig. 3. Specific activity of bound $^{14}$C-CQ in RNA, DNA and protein fractions. Similar results were obtained in several experiments.

of a low pH may lead to a greater cytotoxicity of CQ in different cell systems.

In labelling experiments, both the accumulation of free CQ and the ratio of bound CQ to total CQ uptake increased when the pH was lowered, thus leading to an increase in the amount of CQ bound to the cells. The binding of CQ to DNA at various pHs was more efficient than other macromolecules of RNA and protein, under the same conditions. Lowering of the pH exceedingly enhanced the DNA binding. DNA is probably the main target of CQ as the cytotoxicity of the compound which was enhanced at pH 6 correlated well with the binding to the DNA. Nakamura et al. studied possible biochemical mechanisms related to the action of CQ with leukemic cells and found that binding of this compound to DNA results in an inhibition of the synthesis of both DNA and RNA (8).

The finding that CQ binding to calf thymus DNA in vitro is also enhanced at pH 6 suggests that the intracellular pH becomes acidic to the same extent as the extracellular pH and hence a more extensive binding occurs. However, such can probably be ruled out as there is no concrete evidence that extracellular and intracellular pHs are inter-affected. It is more likely that an activated state of CQ under extracellular acid conditions can be maintained even after penetration of the drug into the cells where the intracellular pH remains neutral.
Anti-cancer drugs with a cytotoxicity which can be enhanced under mildly acidic conditions should be most clinically effective. We are now designing simple and rapid screening methods for such drugs and the descriptions will be made elsewhere.

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