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Anticancer Drug Sensitivity in vitro in the Bladder Cancer Cell Line, KK-47 and Prophylactic Use of Carbazilquinone and Urokinase in Bladder Cancer

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Summary. Using colony formation technique and KK-47 cell line established from a human bladder transitional cell carcinoma, the effects of 6 anticancer drugs, thio-TEPA, Bleomycin, mitomycin C, carbazilquinone, Adriamycin and cis-Platinum, were compared. On the results of tests performed to establish the drug concentration required to achive a 50% inhibition of cell survival, carbazilquinone was chosen for the prevention of recurrences of bladder cancer. The two groups studied consisted of 56 patients (previously untreated group) who were rendered free of tumours by surgical intervention and of 19 patients (thio-TEPA failures group) who had experienced a persistent recurrence of tumours after prophylactic thio-TEPA instillations and were presumed free of the recurrence of tumours after the next surgical intervention. The 2 groups were subjected to prophylactic combined intravesical instillation therapy with carbazilquinone and urokinase. In the previously untreated group, 6 of the 56 patients (10.7%) had a recurrence of tumours, and the recurrence rate after 21 months was 16.7%, using the actuarial method. In the thio-TEPA failures group, 12 of the 19 patients (63.2%) had a recurrence of tumours, a rate at 21 months of 76.1%. A considerable drop in the recurrence rate was obtained by the combined instillation therapy in the previously untreated group. The results in the thio-TEPA failures group suggested the presence of a cross-resistance between both alkylating agents, and of a persistent susceptibility to multifocal lesions. No bone marrow depression was observed but an episode of anaphylactic shock attributable to the use of carbazilquinone occurred in 1 out of a total 75 patients.

Key words: KK-47 cell line, Bladder cancer, Chemosensitivity, Instillation therapy, Carbazilquinone, Urokinase.

INTRODUCTION

In previous papers (4, 5) Hisazumi et al. have studied the effect of combined intravesical instillation of anticancer drugs and urokinase in the postoperative treatment of superficial bladder cancer. An 8-year follow-up study (5) of 54 patients who had undergone the instillation therapy with thio-TEPA and urokinase for 2 years revealed that the total recurrence rates at 1, 3 and 5 years were 10.2, 24.7 and 40.5%, respectively. One of the chief disadvantages of this therapy was the sporadic occurrence of serious leukopenia resulting from the absorption of thio-TEPA through the bladder wall. Since the benefit of the instillation therapy is to allow the use of an appropriate concentration of anticancer drugs under direct exposure conditions, the determination of the drugs which possess a stronger cell-killing effect on in vitro cultured bladder cancer cells may give a clue to improving the chemotherapeutic efficacy in bladder cancer. Although a considerable amount of information is available concerning the influence of the drugs on biomolecular mechanisms and on their pharmacological properties, very little is known on the effect of chemotherapeutic drugs on established bladder cancer cell lines.

The aim of the present study was to assess the cell-killing effects of several anticancer drugs on in vitro cultured KK-47 cells (6) established from a human bladder transitional cell carcinoma and to adopt them in prophylactic intravesical instillation therapy in bladder cancer.

MATERIAL AND METHODS

Anticancer Drugs

Anticancer drugs which have been considered to be concentration dependent in their pharmacologi-

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cal action were used in this experiment. A total of 6 anticancer drugs; thio-TEPA, Bleomycin, mitomycin C, carbazilquinone, Adriamycin and cis-Platinum were studied.

Cell Culture

The KK-47 is a cell line which was established from Stage A, Grade 1 transitional cell carcinoma of the bladder removed from a 50-year-old man, blood group A, in our department during 1977. Its growth, heterotransplantation, microscopic structure and chromosome pattern have been reported by Hisazumi et al. (6). Successive cultures were made on Ham's F 12 medium (Nissin Pharmaceutical Co., Tokyo) supplemented with 20% calf serum (Igaku Seibutsugaku Kenkyusho K. K., Nagoya) and aminobenzyl penicillin in 50 μg/ml at 37°C.

Drug Sensitivity Experiments

In estimating the cytocidal effects of anticancer drugs, an in vitro colony formation technique originated from the single cell technique of Puck and Marcus (13) was employed. KK-47 cells at the 130th passage which had been grown in a semi-confluent monolayer in culture bottles were used for this assay system. They were freed from the glass by gentle trypsinisation and redispersed as a monocellular suspension in the Ham's F 12 medium at a concentration of 60 cells per ml. Triplicate tissue culture dishes, 60 x 15 mm (Falcon, Co., Cal., U.S.A.), were seeded with 300 cells per dish. The dishes were incubated in 5% CO_2 in air at 37°C for 24 h and then each anticancer drug was added to the medium in serially diluted concentrations, in order to determine the survival of the cells under the direct exposure of each of the drugs. The separate exposure time series were done at 2 and 24 h. The dishes were washed 3 times with the Ham's F 12 medium and cultivation was continued for 12 to 13 days to permit macroscopic count of clones. At the end of the cultivation period, the medium was discarded and the clones fixed and stained with Giemsa; after drying, the number of the clones were counted. No drug was added to the control series. This experiment was conducted 5 times per drug, and the mean value and standard deviation for the survival rate were calculated. The drug concentration at 50% inhibition (ID_{50}) was determined from the survival curve.

Clinical Investigation

According to the ID_{50} values of the 6 drugs, carbazilquinone was considered the most effect drug and was therefore applied topically to the bladder in combination with urokinase in order to prevent the recurrence of superficial bladder cancer. The benefit of the combination with urokinase will be mentioned later. For this clinical investigation, two groups of patients were studied. Fifty-six patients who were rendered free of gross tumour by surgical intervention, constituted the previously untreated group. This patient group consisted of 46 men and 10 women, with an average age of 62 and 59 years, respectively. Another 19 patients who had experienced tumour recurrence after initial surgical intervention followed by the prophylactic vesical instillation of thio-TEPA and who were presumed free of the recurrence of tumours after next transurethral resection or partial cystectomy, constituted the thio-TEPA failures group. This failures group consisted of 17 men and 2 women, with an average age of 67 and 64 years, respectively. The distributions of Grade (1), Stage (TNM-Classification) and multiplicity of tumours are shown in Tables 1, 2 and 3. As shown in Tables 1 and 3, the failures group had a significantly higher incidence of tumours of Grade III and of multifocal disease as compared with the untreated group ($p < 0.05$). A solution of 20 ml distilled water containing carbazilquinone (4 mg) and urokinase (24,000 NIH units) was instilled into the bladder cavity once a week for a 5-week period. This therapy was continued once a month during the subsequent one year. Cystoscopic and urinary cytological examinations and urinary FDP estimation were performed every 3 months. Adverse bladder reactions, and peripheral blood counts (white blood cells and platelets) were monitored throughout treatment. Patients were followed for 1 to 20 months (average 9.1) in the previously untreated group, and 3 to 25 months (average 16.1) in the thio-TEPA failures group.

RESULTS

ID_{50} Value

Table 4 shows the ID_{50} values of the 6 drugs. Carbazilquinone showed the lowest ID_{50} value, with ID_{50} being 3.5×10^{-3} μg per ml at the 2-hour exposure and 1.1×10^{-3} μg per ml at the 24-hour exposure. The ID_{50} values for Adriamycin were 3.1×10^{-2} μg per ml and 1.0×10^{-2} μg per ml at the 2- and 24-hour exposures, respectively, one digit higher than those of carbazilquinone. With Bleomycin, cis-Platinum and mitomycin C, the ID_{50} values were 1 to 2 digits higher in order. Thio-TEPA showed almost the same value as Bleomycin at the 2-hour exposure. As carbazilquinone showed the lowest ID_{50} value, it was chosen for the clinical investigation; however, there is no special correlation between the

Table 1. Data according to tumour grade

	Untreated group[a]	Failures group[b]
Grade	No. Pts	No. Pts
I	19	4
II	33	9
III	4	6
Totals	56	19

[a]Mean age 62 years, range 27-84 years

[b]Mean age 67 years, range 38-77 years

Table 2. Data according to tumour stage (TNM-Classification)

	Untreated group	Failures group
Stage	No. Pts	No. Pts
Ta	31	9
T1	19	7
T2	6	3
Totals	56	19

Table 3. Data according to tumour number

	Untreated group	Failures group
No. tumour	No. Pts	No. Pts
Single tumour	40	8
Multiple tumours	16	11
Totals	56	19

Table 4. ID_{50} of anticancer agents in KK-47 cells

	ID_{50} (μg/ml)	
Exposure time	2 h	24 h
thio TEPA	7.6	1.0
Bleomycin	2.4	2.9×10^{-1}
cis-Platinum	1.5	2.9×10^{-1}
mitomycin C	4.6×10^{-1}	2.3×10^{-1}
Adriamycin	3.1×10^{-2}	1.0×10^{-2}
carbazilquinone	3.5×10^{-3}	1.1×10^{-3}

Table 5. Recurrence rate following intravesical combined instillation of carbazilquinone and urokinase (the previously untreated group)

No. cases					Percentage			
Interval following treatment (mos.)	Without recurrence	With recurrence	Without recurrence during interval	Person-mos. exposed	Recurrence rate	Non-recurrence rate	Non-recurrence rate at end of interval	Recurrence rate at end of interval
0- 3	6	3	56	53.0	5.7	94.3	94.3	5.7
4- 6	13	1	47	40.5	2.5	97.5	91.9	8.1
7- 9	9	0	33	28.5	0	100	91.9	8.1
10-12	5	2	24	21.5	9.3	90.7	83.4	16.6
13-15	10	0	17	12.0	0	100	83.4	16.6
16-18	6	0	7	4.0	0	100	83.4	16.6
19-21	1	0	1	0.5	0	100	83.4	16.6

ID_{50} value and the clinically used dosage. Since the usual clinical dose of carbazilquinone is 4 to 6 mg per week, we adopted 4 mg of carbazilquinone in the weekly intravesical regimen.

Incidence of Recurrence

The tumour recurrence rate was calculated at 3-month intervals by the actuarial method (8). As shown in Table 5, in the previously untreated group of 56 patients, there were 3 recurrences during the initial 3-month period, making a recurrence rate of 5.7%. For the second and fourth 3-month periods, recurrence was found in 1 and 2 cases, respectively. Thereafter, until the end of 21-months no recurrence was noted. Thus, the total recurrence rate in the 21 months follow-up was 16.6%. While, in the thio-TEPA failures group which consited of 19 patients, there was a markedly higher recurrence rate (Table 6). For the first 3-month period, recurrence was found in 3 cases, and there were 4 recurrences and 3 recurrences during the second and third 3-month periods of observation, respectively. In addition, there was one recurrence during the fifth 3-month period. The total number of recurrences during the 21 months of observation was 76.1%. The differences between the recurrence rates at 4-6, 7-9, 10-12 and 13-15 .

Table 6. Recurrence rate following combined intravesical instillation of carbazilquinone and urokinase (the thio-TEPA failures group)

No. cases					Percentage			
Interval following treatment (mos.)	Without recurrence	With recurrence	Without recurrence during interval	Person-mos.	Recurrence rate	Non-recurrence rate	Non-recurrence rate at end of interval	Recurrence rate at end of interval
0- 3	0	3	19	19	15.8	84.2	84.2	15.8
4- 6	1	4	16	15.5	25.8	74.2	62.5	37.5
7- 9	0	4	11	11	36.4	63.6	39.8	60.2
10-12	3	0	7	5.5	0	100	39.8	60.2
13-15	2	1	4	2.5	40.0	60.0	23.9	76.1
16-18	0	0	1	1	0	100	23.9	76.1
19-21	1	0	1	0.5	0	100	23.9	76.1

months of the observation intervals in both groups were significant using the Fisher's exact probability test. This suggests that the prophylactic protocol had no significant effect in lowering the recurrence rate in the thio-TEPA failures group.

Side Effects

No bone marrow depression was observed in either group. Irritant action on the bladder with pollakisuria, dysuria and haematuria occurred in 7 out of 56 patients (12.5%) in the untreated group only; the instillation therapy was then continued by another regimen including thio-TEPA instead of carbazilquinone. One of the 7 patients had a sudden onset of lower abdominal pain, cough and shock with a diffuse erythematous rash after vesical instillation of carbazilquinone and he recovered after the administration of hydrocortisone and diphenhydramine. The intradermal challenge test revealed that the causative drug was carbazilquinone.

DISCUSSION

There have been convincing reports (2, 3) regarding high postoperative recurrence rates of low grade superfical tumours in bladder cancer and the close relationship between the recurrence and the presence of areas of unstable epithelium in the apparently normal-looking bladder mucosa. Pavone-Macaluso et al. (12) have reported on the 2-year recurrence rate of approximately 60% after surgical intervention, and Nocks et al. (11) have found recurrence of tumours in 19 of 24 patients rendered free of tumour by transurethral resection alone in a 2-year follow-up study.

Carbazilquinone has carcinostatic groups in its molecule - quinone, aziridine, and urethane-, which is an alkylating agent modified from the parent compound, mitomycin C (10). Concerning ID50 values obtained in the in vitro study using KK-47 cells, carbazilquinone possessed an extremely high toxicity as compared with mitomycin C.

In a pilot experiment (9) regarding enhanced cell-killing by carbazilquinone and urokinase, a combined exposure of urokinase and carbazilquinone to KK-47 cells resulted in a significantly greater decrease of cell growth compared with a single exposure to carbazilquinone. In addition, ^{3}H-thymidine uptakes of the cells were remarkably reduced by the combined exposure. Studies of pharmacological interaction between carbazilquinone and urokinase have revealed no change in action and chemical properties.

On the basis of these results, the present authors adopted the prophylactic clinical trial with carbazilquinone and urokinase in bladder cancer. The previously untreated group showed a significantly lower recurrence rate compared with the thio-TEPA failures group. In other words, the combined instillation therapy with carbazilquinone and urokinase exhibited no significant prophylactic effect in the thio-TEPA failures group. These recurrences of tumours may be considered to have become resistant to the cytotoxic effect of thio-TEPA after repeated instillations. Cross-resistance is common to other alkylaing agents and resistance has been attributed to one of the 3 following mechanisms; 1, decreased uptake, 2, inactivation by increased amounts of nonprotein sulphydryl groups and 3, an increased excision-repair mechanism (7); because of this drug resistance, carbazilquinone may have little effect in lowering recurrence rates in the thio-TEPA failures group. In addition, the high rate of multiplicity of initial tumours in the thio-TEPA failures group (Table 3) may be another reason for the lack of prophylactic effect on multifocal lesions.

It is well known that there is a wide gap between results of the screening test of anticancer

agents with various tumour cells of animals and humans and the results of their clinical application. The discrepancy is considered to be related to the biological difference between strains, to immune states of hosts and pharmacological questions such as drug affinity and absorption, excretion, breakdown and cell-killing kinetics of anticancer agents. To confirm the clinical significance of the drug sensitivities in vitro, an assay system including a number of different bladder cancer cell lines should be used. Follow-up studies must be continued on the combination intravesical regimen in order to learn more of its beneficial effects.

Serious side effects from intravesical instillation of carbazilquinone were found in one case, and slight bladder irritating symptoms were reported in 7 out of 56 patients. Further studies into mechanisms of this shock are neccessary.

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