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阿霉素对重组人肿瘤坏死因子介导的结肠癌细胞(COLO205)细胞毒增敏作用

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【摘 要】目的:评价阿霉素和 rHuTNF 抗人结肠癌细胞株的序贯作用。方法:采用克隆生成试验(Hamburger-Salmon 法),研究 DOX 和 rHuTNF 序贯作用于 COLO205 细胞株后的抗增殖效果。结果:对结肠癌细胞系 COLO205,存在 DOX-rHuTNF 序贯抗增殖作用;多数联合给药情况下出现相加/协同作用。结论:DOX 存在时,TNF 的抗癌作用增强,从而支持 DOX 可干扰细胞保护机制以使癌细胞对 TNF 细胞毒作用更为敏感的理论。

【关键词】肿瘤坏死因子;阿霉素;COLO205;克隆生成试验;药物序贯作用

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Sensitization of colon cancer cells (COLO205) treated with Doxorubicin to rHuTNF-mediated cytotoxicity

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【Abstract】 ***Objective***: To evaluate the sequential action of rHuTNF and doxorubicin (DOX) against human colon cancer cell line (COLO205). ***Methods***: With clonogenic assay (Hamburger-Salmon method), the sequential roles of rHuTNF and DOX on COLO205 were investigated. The dose-effect relationships of each drug alone and in combination were analyzed by examining the survival fraction of the drug combination. ***Results***: The action of DOX and rHuTNF against proliferation was found to be schedule-dependent in colon cancer line COLO205; marked synergistic effects were observed in most instances of treatment, when DOX was administered prior to and not following rHuTNF. ***Conclusion***: The anti-tumor effect of TNF can be enhanced in the presence of DOX, suggestion that the addition of DOX may interfere with the cellular protective mechanism, causing the tumor cells to become more susceptible to the cytotoxic action of TNF.

【Key words】 TNF; Doxorubicin; COLO205; Clongenic assay; Sequential roles of drugs

Tumor necrosis factor (TNF) is a cytotoxic monokine produced by macrophages stimulated with bacterial endotoxin. TNF participates in inflammatory reaction and wound healing, and possesses anti-tumor activity. TNF, which is also called cachectin, can induce septic shock and cachexia. TNF can combine with receptors (R_1/R_2) on selected tumor cells and induce their death and mediate the anti-tumor action of natural cytotoxic cells. The combinations of *rHuTNF* and *DOX*, *5FU*, *melphalan*, as well as bleomycin, have previously shown obvious synergism in enhancing the anti-proliferative effects on human colon cancer cell lines[1].

The present study investigated the role of schdeule-dependence of TNF and DOX on COLO205. Dose-effect relationships of each drug alone and combination were analyzed by examining the sur-

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vival fraction of the drug combination.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Colon Cancer Cell Lines: An established colon cancer cell line, COLO205, was obtained from ATCC. The cell line was matintained in RPMI 1640 (Gibco, Grand Island, NY) with 10% (v/v) fetal calf serum (Flow Laboratories, Newburg Park, CA), 1% (v/v) penicillin - strpetomycin and 1% L - glutamine and kept at 37℃ in a humidified atmosphere with 7% CO_2 and 20% O_2. The cells were devoid of mycoplasma or bacterial infection and passaged serially. Cell lines were grown as monolayers. Tumor cells were harvested during the period of exponential growth. Cell detachment was achieved by incubation with hypoosmotic medium for 15 minutes at 37℃. The cells were then shaken off the bottom of the flask and washed twice in McCoy's 5A at 200 × g for ten minutes. The resulting cells in suspensions were counted using a hemocytometer. Viability, as determined by trypan blue exclusion, was > 90%. The single cells were plated at a concentration of 1×10^4 viable tumor cells per plate in the upper layer of the two layer agar culture system. Plating efficiencies for COLO205 cells were 15.73%.

1.1.2 Drugs used: Recombiant human TNF (rHuTNF) in highly purified form was provided by Genentech Inc. South San Francisco, California. The specific activity of rHuTNF was 5×10^7 U/mg protein. rHuTNF was tested by adding to the agar matrix to permit continuous exposure during the culture period. A minimum of five concentrations were tested (e. g. 10, 50, 100, 500, 1000 U/ml). Appropriate dilutions were made with McCoy's 5A containing 10% heat inactivated fetal bovine serum. Doxorubicin (DOX) was obtained from the Arizona Cancer Center's research pharmacy service. It was kept in −80℃, and thawed in a 37℃ waterbath prior to use. In detailed titrations, rHuTNF and DOX were tested alone and in combination at fixed ratios.

1.2 Methods

Cloning assay: A double layer soft agar assay was used as described previously by Hamburger and Salmon.[2~6] Tumor cells were plated into the upper layer of a double layer agar culture system. The under layer consisted of enriched McCoy's 5A medium with 0.5% agarose. The overlayer consisted of CMRL 1066 medium with tumor cells and 0.3% agarose. The tumor cell concentration used was 1×10^4 cells per plate. Positive and negative control plates were included in all assays, and each drug or both combination was tested in triplicate. Testing was done by continuous exposure to drugs (0.2 ml) added to the upper agarose layer. The plates were then incubated for 6 − 14 days (depending upon the growth characteristics of the tumor cell line), in 7% CO_2, and air at 37℃ in a humidified incubator. Prior to final colony counting, plates were examined with an inverted microscope, and colony formation was evaluated with a viability stain. Colonies of al least 60 cells in diameter were counted with an automated image analysis system (Bausch and Lomb Omnicon FAS II), which had been optimized for tumor colony counts. Results were expressed as the percentage of colony growth compared with the controls.

1.3 Data Analysis[7]

Definitions used in data analysis: Table 1 shows the definitions of synergistic, additive and other effects.

Tab 1 Definitions of drug combination effects

Synergistic	SFa + b < (SFa)(SFb)
Additive	SFa + b = (SFa)(SFb)
Subadditive	SFa + b > (SFa)(SFb)
	< SFb, when SFa > SFb
Antagonistic	SFa + b > (SFa)(SFb)

SF: surviving fraction; a, b: Drugs; a + b: drugs used in combination.

A statistical test for deviation from additive effect was constructed as follows: for a given dose of drug a, surviving fraction of cells, SFa, was observed; likewise, for drug b, we observed SFb. Then we observed SFa + b. If the additive effect held, then SFa + b = (SFa) · (SFb). When single concentrations and their combination were studied, synergism or inhibition was determined for each experiment individually, the P value being 0.05 in comparison to the single additive effect hypothesis.

2 Results

2.1 Cytotoxicity of rHuTNF treatment with pre-incubation of DOX on COLO205 cell line

2.1.1 Effect of sequential treatment of COLO205 cells with DOX and rHuTNF

Tab 2 Effect of sequential treatment of COLO205 cells to DOX (0.001μg/ml) and rHuTNF

Drug	Concentration	Percent Survival With drug alone ($\bar{x} \pm s$)	Percent Expected Survial in combination (additive effect)	Percent Observed Survival in combination ($\bar{x} \pm s$)
rHuTNF	10U/ml	98.5 ±10.4	80.6	66.8±2.7
	50U/ml	100.4±9.1	82.2	65.5±5.1
	100U/ml	67.9 ±4.0	55.6	50.8±4.8
	500U/ml	61.1 ±8.7	50.0	41.1±4.4
81.9±7.0*	1,000U/ml	54.3 ±2.9	44.4	41.3±1.8

* $\bar{x} \pm s$: percent survial of control DOX (0.001μg/ml) pretreated cells.

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After pre－incubation with DOX(0.001μg/ml), the percent Survival rates of pre－treated cells was 81.9±7.0. We obtained the % Survival for continuous exposure to rHuTNF alone, (i.e. 98.5±10.4, 100.4±9.1, 67.9±4.0, 61.1±8.7, 54.3±2.9), as well as % ES(Expected Survival) for drugs in combination(80.6, 82.2, 55.6, 50.0 and 44.4). In these experiments, we obtained the following % OS(Observed Survival) for drugs in combination: 66.8±2.7, 65.5±5.1, 50.8±4.8, 41.1±4.4, and 41.3±1.8, respectively.

2.2.2 Effects of sequential treatment of COLO205 cells with DOX (0.005μg/ml) and rHuTNF

Tab 3 Effects of sequential treatment of COLO205 cells with DOX(0.005μg/ml) and rHuTNF

Drug	Concentration	Percent Survival With drug alone ($\bar{x}\pm s$)	Percent Expected survival in combination (additive effect)	Observed survival in combination % ($\bar{x}\pm s$)
rHuTNF	10U/ml	98.5 ±10.4	77.6	58.1±1.2
	50U/ml	100.4±9.1	79.1	58.2±7.3
	100U/ml	67.9 ±4.0	53.4	54.4±3.0
	500U/ml	61.1 ±8.7	48.1	40.7±1.5
78.7±6.0*	1,000U/ml	54.3 ±2.9	42.7	22.4±8.3

* $\bar{x}\pm s$: percent survival of control DOX(0.005μg/ml) pretreated cells.

Table 3 showed that after pre－incubation with DOX(0.005μg/ml), the % Survival of pretreated cells was 78.7±6.0. We obatined % Survival rates with continuous exposure to rHuTNF alone of 98.5±10.4, 100.4±9.1, 67.9±4.0, 61.1±8.7 and 54.3±2.9. Then we obtained % ES for drugs in combination: 77.6, 79.1, 53.4, 48.1 and 42.7. In our experiment, we found % OS rates of 58.1±1.2, 58.2±7.3, 54.4±3.0, 40.7±1.5 and 22.4±8.3, respectively.

2.2.3 Combined effects of sequential treatment of COLO205 cells with DOX and rHuTNF

Tab 4 Combined effects of sequential treatment of COLO205 cells with DOX and rHuTNF

Treatment	Combined effect Concentration of rHuTNF(U/ml) 10	50	100	500	1000
DOX 0.001μg/ml 1h Then rHuTNF	S	S	S	S	S
DOX 0.005μg/ml 1h Then rHuTNF	S	S	Add	S	S

S: synergism; Add: additive effect. Synergism or addition is $P<0.05$ in comparison to corresponding values of OS and ES.

Tabe 4 showed that anti－tumor effects of rHuTNF were enhanced markedly, after pretreatment of COLO205 cells with different doses of DOX(0.001 and 0.005μg/ml).

3 Discussion

It is well－known that TNF can induce the cytotoxic/cytostatic effects in tumor cells. Tumor cells can show various reactions before death under TNF action, eg. after tumor cells were stimulated by TNF, mRNA/protein synthesis was higher than before. Only when cells were treated for 24h did tumor cells undergo degeneration and nercrosis.

Recently, we learned that there is a protective action(rescue system) in tumor cells. When DOX inhibits cytosolic caspase activity, this "rescue mechanism" would be impaired, and hence the cells would accumulate excessive reactive oxygen－producing mitochondria and would be far more sensitive to TNF－induced necrosis[8,9,11].

First of all, TNF interacts with its receptors, and then cognate interaction with its receptor leads to the formation of toxic products in the cytoplasma. This formation and resulting damage may be prevented by protease inhibitors. Cells may protect themselves from the effects of these toxic compounds by the synthesis of detoxifying enzymes. Our data showed that in the mode of "short (4h) rHuTNF pre－incubation, followed by DOX treatment", rHuTNF can decrease the cytotoxicity of DOX on tumor cells, owing to the production of a protective mechanism followed by cellular

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stimulation of rHuTNF[10]. In the mode of "DOX first, TNF second", DOX can induce increased rHuTNF cytotoxicity, suggesting that the tumor cells, perhaps in the presence of DOX, can no longer make these protecting, detoxifying enzymes and, as a result, are destroyed. Furthermore, DOX could enhance Fas receptor expression on the plasma membrane of colon cancer[9].

In general, the interaction of DOX and rHuTNF was found to be schedule－dependent in colon cancer cell line COLO205. Marked synergism was observed in most instances of treatment when DOX was administered prior to, but not following rHuTNF. rHuTNF kills cancer cells, but also induces a protective mechanism in the cells. However, the anti－tumor effect of TNF can be enhanced in the presence of DOX, Suggesting that the addition of DOX interferes with the protective mechanism, and the tumor cells then become more suseptible to the cytotoxic action of TNF.

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(上接第 392 页)

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