Anti Cervix Cancer Activity of Co-immobilized Tumor Necrosis Factor-α and Interferon-γ

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Tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) are cytokines with strong antitumor activities. They were reacted with a photoactive arylazide-4-azidobenzoic acid, resulting in photoactive TNF-α and IFN-γ. The infrared (IR) spectra of these products showed the characteristic absorption of an azido group at 2127 cm^{-1}. By photo-immobilization, this modified TNF-α and IFN-γ were immobilized on polystyrene membranes for cell culture to prepare biomaterials. The micro-morphology of photoactive cytokines was observed with a scanning electron microscope (SEM). The inhibitory effect on growth of Hela cells and inducing apoptosis activity of these two cytokines were analyzed by growth curve, transmission electron microscope (TEM) and fluorescence active cell sorter (FACS). The results showed that co-immobilization of IFN-γ and TNF-α had significant inhibitory effect on growth of Hela cells, inhibitory rate up to 82%, and IFN-γ had obviously synergistic action.

KEY WORDS: Tumor necrosis factor (TNF-α); Interferon-γ (IFN-γ); Cervix cancer cell line; Photo-immobilization; Polystyrene; Inhibitory activity

1. Introduction

Tumor necrosis factor-α (TNF-α) is a kind of cytokine produced by activated monocytes and phagocytes that could kill many sorts of tumor cells effectively both *in vivo* and *in vitro*[1]. It has been demonstrated that TNF-α is able to mediate many biological effects such as cell proliferation and differentiation, while it results in cell death through initiation of apoptosis mechanism[2].

Interferon-γ (IFN-γ) is a sort of important cytokine of which over twenty kinds have been separated and identified recently. It is a group of macromolecule glycoprotein with similar structure and closed function produced by immunocytes through anti-virus response, while the organ is infected by viruses. IFN-γ has several biological actions including broad-spectrum of anti-virus, anti-proliferation and immune regulation in cells of the same kind. It is a kind of important anti-virus and anti-tumor medicine indicated by clinical application trials for more than 10 years. Moreover, IFN-γ could improve cell apoptosis induced by TNF-α[2].

Photo-immobilized biological material is a kind of hybridization biological material connected to biological system synthesized by photo-chemical-immobilization that couples the active species with polymeric vectors firmly. The biological consistency of such material is fairly ideal, compared with the formal physical and chemical methods that altered material surface property. Almost all sorts of compounds (natural/synthesized) including hydrophilic and hydrophobic high polymers, anticoagulant, antibacterial, antibiotics, growth factors, peptides, enzyme, protein and other biological molecules beneficial to human could be immobilized to biological medical macromolecule material surface by photo-chemical-immobilization and prepared to biological materials with various actions and purposes[3].

In the present study, Hela cells cultured on 24-well polystyrene film immobilized with cytokines (IFN-γ, TNF-α) were adopted and the effect of IFN-γ and TNF-α co-immobilization on Hela cells growth was studied. It was the first time to investigate the Hela cells apoptosis induced by chemical co-immobilization of IFN-γ and TNF-α although the anti-tumor coordination of these two cytokines has been reported already.

2. Experimental

2.1 Reagents and instruments

Recombinant human IFN-γ was purchased from Lizhu Biotech Pharmaceutical Co. (Zhuhai, China); recombinant human TNF-α was from Saida Biotech Pharmaceutical Co. (Shanghaii, China); dimethylformamide (DMF), N-(4-azidobenzoyloxy) succinimide was from Sigma; 24-well culture plate was from Biofil co.; RPMI 1640 and trypsin were from GIBCOBRI; new born calf serum (NBCS) was from Sijiqin Biotech Material Co. (Hangzhou, China); HY-5 timing multi-purpose vibrator (Jintan, Jiangsu, China); hyper-velocity refrigeration centrifuge (Mikro22R, BECKMAN Corperation); ultraviolet spectrometer (SHIMADZU UV2450 Japan); refrigeration evaporator (25SL) (VIRTIS COMPANY INC,USA); XL-30E SEM scan electronic micrograph (Phlips, Holand,); WQF-300 Fourier transform infrared spectrometer (the Second Optician, Beijing, China); SPM-3000 atom-powered micrograph (Benyuan, Beijing, China) were used in this study.

2.2 Synthesis and detection of photoactive cytokine

TNF-α (5 μg, 2.94×10^{-7} mmol) was added to the solution of N-(4-azidobenzoic acid radical)-succinimide with DMF/PBS (phosphate-buffered saline solution) (pH 7.4) (4:1, 2 ml) under stirring in ice bath. Then, let the solution react at 4°C. After synthesis, TNF-α derivactive with azidophenyl was purified by ultra-filtrate film (Milipore Molecut II, 5kDa) and evaporated by freeze-drying.

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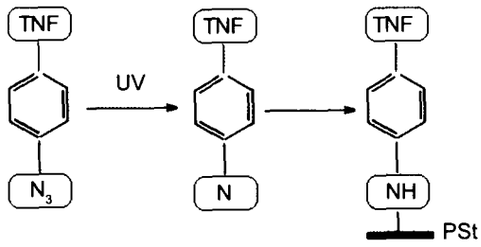

Fig.1 Schematic diagram of photochemical reaction upon UV light radiation

In the end, infrred (IR) analysis of TNF-α (control) and photosensitive TNF was performed.

2.3 Photo-immobilization[4~13]

The photo-immobilization process of TNF is shown in Fig.1. IFN-γ was added into six 24-well polystyrene tissue culture plates (A-F) in various concentrations. In group A and B, IFN-γ was re-immobilized in the same concentration on the basis of TNF-α immobilization (5~50 ng/well), six parallel wells for each concentration. In group C, D, E and F, TNF-α and IFN-γ are immobilized on plates in the concentration of 10~100 ng/well, respectively. Six parallel holes were made for each kind of density and six parallel holes for the blank comparison. Afterward, those plates were freeze dried and irradiated at 2 cm under ultraviolet lamp (4 W) for 15 min. Then IFN-γ was immobilized on polystyrene material for the extremely active character of azides. After photo-immobilization, those plates were washed by PBS (-) (pH 7.4) solution repeatedly until the ultraviolet absorb of leaky PBS (-) is zero. Finally, those plates were evaporated in refrigerator naturally.

2.4 Morphological observation of photo-immobilized TNF-α by SEM

Plates in F group were observed by SEM and blank control was performed simultaneously. Those plates were dried before ice air and gilt to be observed clearly.

2.5 Secondary culture of human cervix cancer cells

Human cervix cancer cells line Hela was provided by the Experimental Animal Centre of Zhongshan University of Medical Sciences. Human cervix cancer cells were secondary cultured with RPMI-1640 culture medium that contains 10% fetal serum, 0.03 mg/ml penicillin and 0.05 mg/ml streptomycin, pH 7.0~7.4. Hela cells were digested with PBS (-) containing 0.25% (w/v) trypsinase and 0.02% (w/v) Ethylenediaminetetraacetic acid (EDTA) and were washed with serum culture medium. Hela cells were inoculated in 15 ml culture bottles and cultured in 5% CO_2 culture cases at 37°C. Then they were transferred into 24-well polystyrene plates and cultured continually after being passaged to be vigorous.

2.6 Inhibition trials of photo-immobilized TNF-α, IFN-γ and free TNF-α, IFN-γ on human cervix cancer cells growth

Those 24-well polystyrene plates immobilized with cytokines in various concentration (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ng/well) were sterilized by 70% (v/v) ethanol and the left ethanol was washed by sterilized water and were added with 1 ml non-serum medium per well after airing. Another 24-well culture plate labeled with G was adopted and added 100 ng free IFN-γ, TNF-α, IFN-γ+TNF-α. Six parallel holes were made for each sample and 1 ml non-serum was added to each hole.

Vigorous Hela cells were adopted and digested by above method after removal formal serum medium and were suspended in non-serum medium and adjusted to 10×10^5/ml by counting. Then the cells were added into 24-well plates with 0.1 ml per well and some other cells were cultured in original non-serum medium as a control. After culture in 5% CO_2 culture cases at 37°C for 48 h, the cells were counted with hematocyte counter plate and were investigated by SEM and flow cytometer (FCM) on study of anti-proliferate and apoptosis inducement mechanism *in vitro*.

2.7 Detection of cell cycle distribution and identification of cell apoptosis by FCM[14]

The Hela cells processed by IFN-γ, TNF-α, IFN-γ+TNF-α (10~100 ng/well) and those from control were digested by pancreatin and washed by PBS and collected in centrifuge tubes. The supernatant was removed after centrifugation at 1500 r/min for 5 min and the precipitated cells were suspended gently and separated evenly in 90% cold ethanol by straw, and immobilized in refrigeratory for more than 12 h. Before measurement, the ethanol was eliminated by centrifugation and the cells were washed by PBS again. Then 1500 μg PI (propylphridine iodide) was added after removal of ethanol by centrifugation and the cells were operated for more than 15 min. To guarantee monocelled suspension, the samples were filtrated with 200 ocular nylon net. Analysis of DNA cell cycle and apoptosis were performed by FCM.

2.8 Execution of TEM samples of Hela cells[15]

Suspended Hela cells processed by IFN-γ+TNF-α (10~100 ng/well) for 48 h were centrifuged at 1500 r/min for 5 min and washed by PBS for one time. After removal of supernatant, the precipitated cells were immobilized by 0.25% neutral glutaraldehyde for TEM slice. Meanwhile, the normal cells were centrifuged and immobilized after being digested by 0.25% pancreatin as control.

3. Results and Discussion

3.1 Infrared spectrogram of TNF-α

TNF-α, whose molecular weight is 17 kilodalton, is composed of 157 amino acid residues in which the free residues could undergo nucleophilic substituent reaction with active carboxyl and form amide bond that causes TNF-α chain modified by azide residues. All the modified sites locate in nitrogen residues of lysine because of the higher reactivity of aromatic azide components with lysine and the lower reactivity with other amino acid residues. The absorb peak of azide emerged at 2126.59 cm^{-1}, indicating that TNF-α has been modified by azide residues and photo-reactive TNF with azide residues has been synthesized.

3.2 Morphological observation of photo-immobilized TNF-α by SEM

Photo-chemical-immobilization, employed to alter surface property of biological medical macromolecule

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Table 1 Influence of Hela cells growth by IFN-γ single immobilization

IFN-γ concentration /(ng/well)	Number or cells /$\times 10^4$	Inhibition Rate /%
0 (control)	3.76±0.44	–
10	3.13±0.36	17
20	2.69±0.47	29
30	2.75±0.31	27
40	2.63±0.25	30
50	2.54±0.24	32
60	1.58±0.34	58
70	2.00±0.42	47
80	2.25±0.35	40
90	2.75±0.50	27
100	2.42±0.49	36

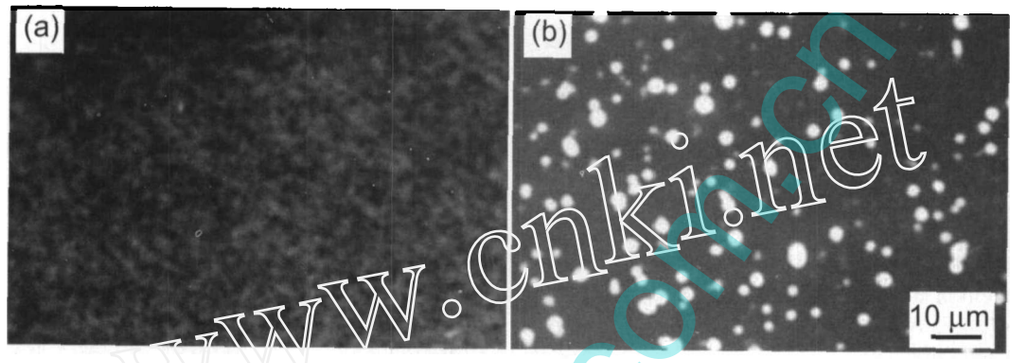

Fig.2 Micrographs of the polystyrene surface (a) and polystyrene surface photo-immobilized with TNF-α (b)

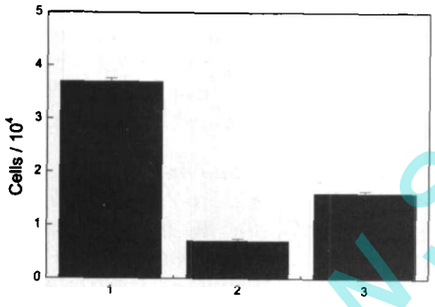

Fig.3 Numbers of Hela cells in the presence: 1—control, 2—photo-immobilized TNF-α+IFN-γ (20 ng), 3—TNF-α+IFN-γ (20 ng) after 48 h culture

materials, is a kind of method that couples biological molecules with special properties to material surface by utilizing ultraviolet or visible light. Its mechanism is to apply chemical connective components with thermoactive and photo-active radicals (aromatic azide and diphenylephrine) to couple some kinds of compounds to material surface to change surface characteristics. The biological reactivity and pharmaceutical effect stability of protein medicine will be enhanced by immobilization. Bioactive molecules coupled with covalent bonds will be well-ordered on material surfaces through photo-immobilization.

Figure 2(a) shows micrographs of the polystyrene surface observed by SEM. Figure 2(b) displays the polystyrene surfaces after being photo-immobilized with TNF-α. These figures demonstate a layer of compact and even protein formed on the polystyrene surface after TNF-α immobilization that indicates TNF-α was well-ordered on polystyrene surface through covalent bonding.

3.3 Influence of immobilized IFN-γ and co-immobilization (IFN-γ+TNF-α) cooperation on cells growth curve of Hela cell

It was noted that many human cervix cancer cells in culture wells exfoliated and changed into round shape and had vacuoles interiorly as the time was prolonged after being cultured on 24-well polystyrene plates co-immobilized with TNF-α+IFN-γ and single immobilized with IFN-γ for 48 h. The cells clones in immobilized wells were smaller and those of blank control were larger. Moreover, the cells in control were full on the wall of wells that had little individual round cells.

For single immobilization, the inhibitory effect of Hela cells by IFN-γ was not significant at low concentration and its inhibition rate was only 29% at concentration of 20 ng/well. The inhibitory effect increased gradually as the elevation of concentration and the inhibition rate reached 58% at concentration of 60 ng/well (Table 1).

For co-immobilization, the inhibitory effect was better at the concentration of 20 ng/well with inhibition rate of 82%. It was presumed that the major factor that inhibited Hela cells at concentration of 20 ng/well was TNF-α. Co-immobilization also had preferable inhibition effect at 60 ng/well with inhibition rate of 73% (Table 2) and the primary one of inhibition presumably was IFN-γ.

3.4 Comparison of inhibitory rate of immobilized and natural TNF-α, IFN-γ on human cervix cancer cells

As shown in Fig.3, the inhibition rate of Hela cells

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Table 2 Influence of Hela cells growth by IFN-γ+TNF-α co-immobilization

IFN-γ+TNF-α concentration /(ng/well)	Number of cells /$\times 10^4$	Inhibition rate /%
0 (control)	3.76±0.44	–
10	2.75±0.36	27
20	0.67±0.29	82
30	2.08±0.41	45
40	3.25±0.83	14
50	1.42±0.29	62
60	1.00±0.35	73
70	2.25±0.64	40
80	1.99±0.35	47
90	1.75±0.53	53
100	1.83±0.33	51

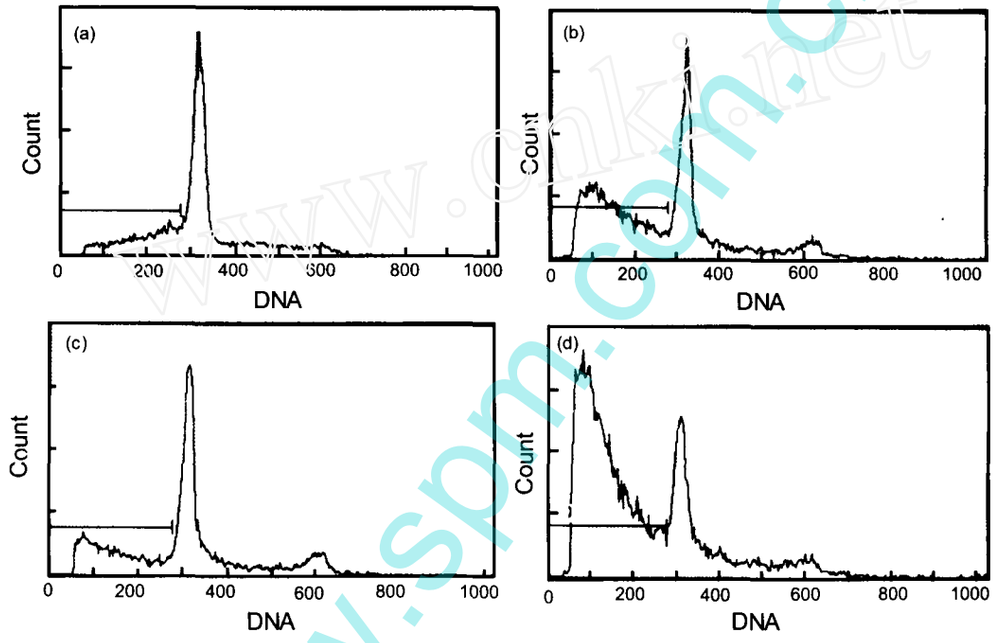

Fig.4 Effect on the cell apoptotic peaks of Hela cells: (a) control, (b) TNF-α, (c) IFN-γ, (d) IFN-γ+TNF-α

by immobilized TNF-α+IFN-γ (20 ng) was about 82% (as 2) that was higher than the natural (as 3); Such a result was consistent with that of another study of which immobilized insulin, epithelial growth factor and TNF acted more markedly than solvent even in small quantities. Immobilized cytokines had not only high inhibitory action, but also slow and persistent power. It is believable that the inhibitory action of immobilized cytokines will the free with prolonging the time.

3.5 Influence of TNF-α, IFN-γ and TNF-γ+IIFN-α on the cell apoptosis peaks and cell cycle of Hela cells

As shown in Fig.4, typical apoptosis cell peak (sub-peak) presented in the histogram after processed by IFN-γ, TNF-α, IFN-γ+TNF-α. Peak in Fig.4(a), the blank control, was quite little although higher than theoretic. The probable reason is that more suspended cells were adopted and most of them had been apoptosis. Figure 4(b), (c) and (d) refer to the apoptosis peak effected by TNF-α, IFN-γ and TNF-α+IFN-γ, respectively. The highest one was co-immobilization (TNF-α+IFN-γ) that was the most powerful to induce apoptosis and TNF-α was superior to IFN-γ.

3.6 Morphological observations of normal and necrotic Hela cells and that processed by TNF-α+IFN-γ

As shown in Fig.5(a), cytomembrane and nucleus membrane in normal Hela cells were complete and double-unit was presented. The organelle shape and mitochondria cristae were also integrated. There were many rough surfaced endoplasmic reticulum and ribosome in cytoplasm. Most of the euchromatins in nucleus were white and the heterochromatin interspersed among nucleoplasm was in spot shape. The nucleus/cytoplasm ratio was 1:1. The shape of chromatin in necrotic cells was irregular gobbet that did not focus at nucleus margin as that of apoptosis (Fig.5(b)). The structure of cytomembrane, nucleus membrane and organelle were disturbed and there were vacuoles in mitochondria. Cytoplasm and its contents were discharged when the cells were disrupted.

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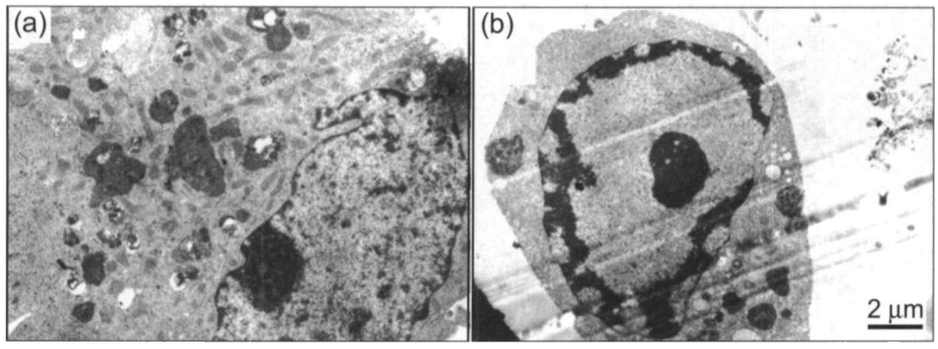

Fig.5 Morphological observation of Hela cells by TEM: (a) normal, (b) apoptotic

As shown in Fig.5(b), the volume of apoptosis Hela cells was 50% of the normal although they had the completed cytomembrane, nucleus membrane and organell membrane. The nucleus/cytoplasm ratio increased; the nucleoplasm was compact and the chromatin was augmented. The chromatin condensed in plaque shape, or concentrated at the nucleus margin as lunular.

4. Summary

Immobilized IFN-γ and TNF-α had more significant inhibitory effect than free cytokines on the growth of adjacent reliant cells. It has been addressed in many reports that immobilized cytokines and insulin inhibited cell growth of adjacent reliant cells more markedly than those in free state. Our former investigation discovered that immobilized active algae-blue protein improved growth of human hepatocarcinoma 7402 cells more effectively than natural one[3]. Subsequently, after a great deal of studies, it is considered that the immobilized active factors could escape from being endocytotic and wasted by cells that guaranteed them function with receptors in cellular membrane for a long time. We also considered that the enhanced anti-cancer activity of photo-immobilized IFN-γ and TNF-α was caused by the prolonged contact between active protein and relative receptors in cancer cells. Moreover, in co-immobilization of IFN-γ+TNF-α, the capacity of killing cancer cells was improved because of coordination of IFN-γ with TNF-α.

About the mechanism[16~20] of the enhanced anti-tumor effect after coordination of IFN-γ and TNF-α, some investigators regarded that resulted from the increased sensitivity of target cells to TNF as the increase of TNF receptors in target cellular membrane up-regulated by IFN-γ[16]. The difference of target cell cycle of IFN-γ and TNF-α promoted their execution of target cancer cells[17].

The present article revealed that the inhibitory effects of natural IFN-γ, TNF-α, and IFN-γ+TNF-α on Hela cells were inferior to their immobilized and that of single IFN-γ immobilization was inferior to co-immobilization of IFN-γ+TNF-α. Co-immobilized IFN-γ could enhance killing effect on Hela cells through coordination with TNF-α at a certain concentration. The influence of IFN-γ, TNF-α and IFN-γ+TNF-α on cell cycle resulted from the inhibition on cell proliferation through transformation inhibition from S to G_2/M course and the inducement of apoptosis. The typical apoptosis peak (sub-peak) presented in the analysis figure of FCM. It was observed that the heterochromatin in apoptosis cells increased and focused at margin of nucleus membrane by TEM. The coordinative effect of co-immobilized IFN-γ and TNF-α put forward the internal relation among anti-tumor and anti-virus therapy. The carrier medicine made of these two cytokines is potentially applicable to cure cervix cancer.

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