Basic Research Paper

Inhibition of lung cancer cell proliferation by CD40 signaling through tumor necrosis factor I

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Key words: CD40, lung cancer, NCI-H460 cells, A549 cells, Tumor necrosis factor receptor, cell proliferation

Background and Objective: CD40 signaling induces growth inhibition in some tumor cells in vitro, but the precise molecular mechanism remains unclear. This study was to investigate the biological effects and mechanisms of CD40 stimulation on proliferation of lung cancer cell lines NCI-H460 and A549, changes in tumor necrosis factor receptors (TNFRs) and membrane tumor necrosis factor alpha (mTNF-α). Methods: The expression of CD40 on the cell surface, and changes in TNFR and mTNF-α expression after CD40 stimulation were detected by the immunofluorescence technique and flow cytometry. Changes in protein contents of TNFR as well as mTNF-α expression after CD40 stimulation were measured by western blot. The cell proliferation rate was determined by MTT assay. The content of soluble TNF-α(sTNF-α) in the supernatant of lung cancer cells was measured by ELISA assay. Results: The expression rates of CD40 in NCI-H460 and A549 were (89.0 ± 3.2)% and (62.2 ± 4.5)%, respectively. After 48 h of CD40 stimulation, the expression rates of TNFRI in NCI-H460 and A549 became significantly higher [(36.2 ± 4.6)% and (38.5 ± 5.9)%] than those in the corresponding control cells [(15.2 ± 3.1)% and (7.2 ± 1.9)%] (p < 0.05); while the expression rates of TNFRII were significantly lower than those in the control cells [(18.0 ± 1.6)% and (5.8 ± 1.2)% vs. (58.1 ± 3.6)% and (38.8 ± 4.3)%] (p < 0.05); the expression rates of mTNF-α decreased in the two cell lines [(8.7 ± 1.1)% and (7.0 ± 0.9)%] as compared to those in control cells [(15.0 ± 2.1)% and (26.5 ± 3.2)%] (p < 0.05). The level of TNFRI protein was elevated with the downregulation of TNFR as well as mTNF-α in vitro. Conclusion: CD40 signaling inhibits the proliferation of CD40-positive lung cancer cells through mTNF-α/TNFRI in vitro. CD40 is a transmembranous protein with a molecular weight of 48k, and is a member of the tumor necrosis factor receptor (TNFR) superfamily. It is expressed in antigen presenting cells, endothelial cells and some tumor cells.1,2 Studies show that CD40 signaling can inhibit proliferation of tumor cells expressing positive CD40 and some B-cell malignant tumor cells in vitro,3,4 though the mechanisms remain unclear. This study used two strains of lung cancer cells with positive expression of CD40 to investigate the influence and mechanism of CD40 signaling on the cell growth, expression of surface TNFR and membranous TNF-α (mTNF-α), and the biological behaviors of lung cancer cells.

Materials and Methods

Reagents and equipments. RPMI-1640 culture medium (Gibco Company, USA), fetal calf serum (FCS) (Hyclone Company, USA), cell culture plates (Costar Company, USA), and tetrazolium salt (MTT) (Sigma Company, USA) were purchased. Anti-human CD40 monoclonal antibody (5C11) was prepared by the Institute of Medical Biotechnology of Soochow University. Enzyme labeling instrument 550 (Biorad Company, USA), EPICS-ALTRA flow cytometer (Beckman Coulter Company, USA), rat anti-human TNFRI and TNF-α blocking monoclonal antibodies (Hycult Biotechnology Company, Netherland), and phycoerythrin-labeled (PE) rat anti-human IgG control antibodies (Immunotech Company, France) were used. Rabbit and goat anti-IgG control antibodies were bought from Santa Cruz Company (USA), and human TNF-α ELISA test kit was the product of R&D Company (USA).

Cell line and cell culture. Lung cancer cell lines, NCI-H460 (large cell carcinoma) and A549 (adenocarcinoma), were purchased from Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 culture medium supplemented with 10% FCS at 37°C and an atmosphere of 5% CO₂. Cells were passaged every three to four days. Before each experimental trial, cells
A549 and H460 cells, 5 x 10⁵ cells/ml, were seeded in six-well plates and incubated with 5C11 (10 µg/ml) or the control IgG (10 µg/ml) at 4°C for 30 min. After a rinse with PBS, PE-labeled secondary antibodies were added, and incubated for another 30 min at 4°C. After being washed by PBS twice, cells were re-suspended and measured for the expression of CD40 on the cell surface by FCM. The expression rate of greater than 85% was regarded as high expression, 30–85% as moderate expression, and less than 30% as low expression.

Expressions of surface TNFRII and mTNF-α measured by FCM. A549 and H460 cells, 5 x 10⁵ cells/ml, were seeded in six-well plates and incubated with 5C11 (10 µg/ml) for 12, 24 and 48 h. Negative control was set up for each time point. Cells were harvested and PE-labeled antibodies for TNFRI, TNFRII, and TNF-α were added. After incubation for another 30 min at 4°C, cells were washed with PBS and re-suspended before analysis by FCM. The expression rate of greater than 85% was regarded as high expression, 30–85% as moderate expression, and less than 30% as low expression.

Protein contents of TNFRII and mTNF-α measured by western blot. A549 and H460 cells were treated with 5C11 (10 µg/ml) for 0, 12, 24 and 48 h. Cells were lysed with lysis buffer to extract proteins. Based on the Bradford method, protein concentration was measured and protein was separated by SDS-PAGE electrophoresis (80V, 30 min; 120V, 90 min). Protein was transferred to a nitrocellulose membrane and sealed with TBST containing 5% skim milk for 2 h. Rat anti-human TNFRII and mTNF-α primary antibodies (1:200) were added respectively. The membrane was incubated overnight at 4°C and washed by TBS for three times. Then goat anti-rat secondary antibodies (1:2000) were added, and incubated for another 2 h at room temperature. After three times of rinse, the x-ray film was developed in dark room. The targeted proteins were semi-quantitatively analyzed using the UVIDoc imaging device.

MTT assay. A549 and H460 cells were seeded into a 96-well plate (1 X 10⁵ cells/well) and cultured in 200 µl RPMI-1640. Cells were divided into seven groups: (1) blank control group (200 µl RPMI-1640); (2) control group (1 x 10⁵ lung cancer cells were cultured in 200 µl RPMI-1640); (3) 5C11 (10 µg/ml) group; (4) 5C11 (10 µg/ml) and TNFRI mAb (5 µg/ml) group; (5) TNFRII mAb (5 µg/ml) group; (6) 5C11 (10 µg/ml) and TNF-α mAb (5 µg/ml) group; (7) TNF-α mAb (5 µg/ml) group. Six replicate wells were performed per sample. Cells were cultured for 48 h. At 4 h before terminating culture, 20 µl MTT (5 mg/ml) was added into each well. After cell culture, the supernatant was discarded and 100 µl dimethylsulfoxide (DMSO) was added into each well. After the formazan granules were completely dissolved, A value of each well was measured at 490 nm, and the corresponding cell proliferation rate and proliferation inhibition rate were calculated. Cell proliferation inhibitory rate = (A value of the control group – A value of the experimental group) / A value of the control group x 100%. Cell proliferation rate = (A value of the experimental group – A value of the control group) / A value of the control group x 100%.

ELISA. A549 and H460 cells, 2 x 10⁵ cells/well, were placed in a 24-well plate and cultured in 1 ml RPMI-1640. After incubation with 5C11 (10 µg/ml) for 12, 24 and 48 h, the supernatant was collected. The content of sTNF-α in the supernatant was measured according to the instructions of the TNF-α ELISA test kit.

Statistical analysis. Each experiment was repeated three times. Laboratorial data were analyzed using SPSS 13.0 software and presented as mean ± SD. The intergroup comparison was performed by variance analysis, while comparison between two groups was performed by t-test. p < 0.05 was considered statistically significant.

Results
Expressions of CD40 in lung cancer cell lines. CD40 molecules were strongly and moderately expressed on the surfaces of NCI-H460 and A549 cells, with the expression rates of (89.0 ± 3.2)% and (62.2 ± 4.5)%, respectively.

Influence of CD40 stimulation on expressions of TNFRI/II and mTNF-α in lung cancer cell lines. TNFRI and mTNF-α were weakly expressed on the surface of NCI-H460 and A549 cells, while TNFRII was moderately expressed. After CD40 stimulation, the expression of TNFRII was elevated, while those of TNFRII and mTNF-α were reduced (p < 0.05). Phenotypic changes in TNFRII and TNFRII were time-dependent, which were most apparent in the first 24 h (p < 0.05). The downregulation of mTNF-α was most significant in the first 12 h and no changes were observed after 24 h (p>0.05) (Table 1). Comparisons among negative control groups of these three phenotypes in NCI-H460 and A549 at different time points showed no statistical significance (p > 0.05).

CD40 stimulation enhanced the protein content of TNFRII and decreased that of TNFRII in NCI-H460 and A549 cells, but did not affect the protein expression of mTNF-α (Figs. 1–3).

No sTNF-α was found in the supernatant of NCI-H460 and A549 cells.

Effect of CD40 stimulation on the proliferation of lung cancer cell lines. After incubation with 5C11 for 0, 12, 24, 36 and 48 h, the proliferation of NCI-H460 and A549 cells were significantly decreased as compared to control cells (Fig. 4).

Inhibition of TNF-α signaling on proliferation of lung cancer cell lines. Incubation with the blocking antibody of TNF-α significantly reduced the proliferation, while incubation with the blocking antibody...
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Influence of blocking TNF signaling on inhibition of proliferation in lung cancer cell lines by CD40 stimulation. After NCI-H460 and A549 cells were co-cultured with the blocking monoclonal antibody of TNFRI or TNF-α mAb, 5C11 no longer inhibited the proliferation of tumor cells (p > 0.05) (Table 3).

Discussion

CD40 signaling pathway has been found to play an important role in the immune response to tumors both in vivo and in vitro.3-5 Activation of CD40 can directly inhibit tumor proliferation and indirectly enhance the immune response by increasing the sensitivity of tumor cells towards chemotherapy and radiotherapy, upregulating antigen presenting capacity, and promoting maturation of dendritic cells (DCs).5 These provide new approaches for the immunological treatment of lung cancers.

TNFR exists on the surface of many normal and tumor cells. There are two subtypes of TNFR, TNFRI and TNFRII. The molecular weight of TNFRI is 50ku and it is a transmembranous protein with a death domain. It primarily mediates apoptotic signals and can inhibit cell proliferation. It is reported that the CD40-mediated cytotoxic effect is achieved through activation of TNFRI by intrinsic TNF-α and the stimulated CD40 works synergistically with TNFRI-mediated cytotoxic reaction.6 Different from TNFRI, TNFRII does not contain a death domain. Inhibition of TNFRII can induce an increase of apoptosis inhibitory protein FLICE-inhibitory-protein (FLIP) to prevent apoptosis. However, inhibition of TNFRII can cause a decrease of FLIP and promote apoptosis.7 Tanimura et al.8 reveal the TNF-α/TNFRII pathway could activate NF-αB, MAPK, and Akt to induce secretion of matrix metalloproteinase (MMP)-9, thus to increase the infiltrative capacity of cholangiocarcinoma

Table 2  Changes in proliferation of NCI-H460 and A549 cell lines after blocking TNF signaling

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control group</th>
<th>TNF-α mAb group</th>
<th>TNFRI mAb group</th>
</tr>
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<tbody>
<tr>
<td>A549</td>
<td>1.26±0.024</td>
<td>0.905±0.041</td>
<td>28.07±1.74</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>1.67±0.014</td>
<td>1.437±0.011</td>
<td>17.70±1.45</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. control group
cells. Therefore, the TNFRII pathway is important in regulating proliferation and infiltration of tumor cells. As the common ligand of TNFRI and TNFRII, TNF-α exists in two active forms, which are mTNF-α of 26 ku and secretory TNF-α (sTNF-α) of 17 ku. mTNF-α is the precursor of sTNF-α, which becomes sTNF-α after being hydrolyzed by metalloproteinases, such as TNF-α converting enzyme (TACE). sTNF-α mainly binds to TNFRI to transduct signals; while mTNF-α is more closely related to TNFRII, which allows it to bind to and activate TNFRII more easily than sTNF-α. Our previous studies have shown that activation of CD40 could induce phenotypic changes in TNFR, FasL, CD54, CD49, and so on, on the surface of lung cancer cells with positive CD40, and also significantly inhibit proliferation in vitro.11

This study found that TNFRI and TNFRII were weakly and moderately expressed on the surfaces of NCI-H460 and A549 cells. CD40 stimulation increased the expression rate and protein content of TNFRI in NCI-H460 and A549, while decreased those of TNFRII. These suggest that CD40 signaling could regulate the expression of TNFR family in lung cancer cells. CD40 stimulation significantly inhibited proliferation of NCI-H460 and A549 cells, both of which expressed positive CD40. When TNFRI and TNF-α were blocked, CD40 stimulation did not inhibit proliferation of tumor cells. This indicates that CD40 exerts its inhibition on lung cancer cells through mTNF-α/TNFRI. As no sTNF-α was detected in the supernatant of tumor cells, we suppose that lung cancer cells may regulate proliferation through an autonomous pathway of mTNF-α/TNFRI, in which TNFRII signaling mediates the inhibitory signals on proliferation. This is in accordance with the report by another group. Additionally, we detected moderate expression of TNFRII and weak expression of TNFRI in lung cancer cells. During the tumor growth, mTNF-α/TNFRII is probably dominant over TNFRII in maintaining a strong proliferating ability of tumor cells. We also noticed that CD40 stimulation upregulated TNFRI and downregulated TNFRII in lung cancer cells, and inhibition of mTNF-α significantly prevented the proliferation of lung cancer cells. These imply that inhibition of cell proliferation mediated by CD40 is achieved through increasing TNFRI and decreasing TNFRII. CD40 stimulation caused a certain decrease of mTNF-α on the cell surface, even though its protein content remained unchanged. The increased expression of TNFRII and decreased expression of TNFRII was not associated with the change of mTNF-α. The effect of mTNF-α/TNFRII pathway became more apparent to increase TNFRII-mediated inhibition on cell proliferation.

In summary, CD40 stimulation regulates the expression of TNFR family in lung cancer cells with positive expression of CD40, as well as inhibits proliferation of tumor cells in vitro. The inhibition effect is mediated by the mTNF-α/TNFRI pathway. The molecular mechanism of change in TNFR expression in tumor cells and the transduction pathway in inhibiting cell proliferation induced by CD40 stimulation require further research.

### Table 3

Influence of CD40 signaling-mediated proliferation inhibition on NCI-H460 and A549 cell lines after blocking the TNF signaling

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TNF-α mAb (%)</th>
<th>5C11+TNF-α mAb (%)</th>
<th>TNFRI mAb (%)</th>
<th>5C11+TNFRI mAb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H460</td>
<td>17.70±1.45</td>
<td>15.73±1.40</td>
<td>17.92±1.37</td>
<td>18.90±1.41</td>
</tr>
<tr>
<td>A549</td>
<td>28.07±1.74</td>
<td>33.00±2.04</td>
<td>27.56±1.60</td>
<td>27.67±1.71</td>
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</tbody>
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### Acknowledgements

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### References