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Establishment of the New Adjuvant Therapy using CD40-CD40ligand Stimulation after Surgery for Lung Cancer

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Introduction

CD40, which is a member of the tumor necrosis factor receptor (TNFR) family, has been recognized on B cells, dendritic cells, and monocytes. CD40 engagement by C40 ligand (CD40L) plays an important role in the development and function of B cells and dendritic cells, and in the activation, proliferation and differentiation of normal cells. Although the function of CD40-CD40L signaling in malignant neoplasms has been studied, much remains unknown. The goal of this study was to determine the effect of CD40-CD40L stimulation on tumor cell proliferation and its molecular mechanisms in lung carcinomas and to establish a new adjuvant therapy using CD40-CD40L stimulation.

Materials and Methods

Five human lung cancer lines were tested in this study: Calu3 and NCI-1355 were derived from adenocarcinomas, NCI-460 and 1155 are derived from large cell carcinomas, and Sq-1 was derived from a squamous cell carcinoma.

A methyl-thiotetrazol (MTT) assay was used for evaluation of the effect of CD40L with or without interferon gamma (IFN-γ) on the growth of the tumor cell lines. CD40L (1μg/ml) and/or INF-γ (100U/ml) were added in some wells during the assay. 72 hours later, an MTT assay was performed. Values in control groups (non treatment groups) are presented as 100%.

A 5-bromo-2’-deoxyuridine (Brd-U) flow cytometry method was used for cell cycle analysis after 72 hours of incubation with or without CD40L (1μg/ml).

A polymerase chain reaction (PCR) - based cDNA subtraction method (subtraction PCR method) was used to identify genes expressed through stimulation of CD40 signaling on the Calu3 and NCI-460 cell lines.

A real-time quantitative reverse transcriptase-PCR (real-time PCR) analysis was performed to verify whether the genes identified with the subtraction PCR method were actually up-regulated.

Results

Three of the five cell lines, Calu3, NCI-460, and Sq-1, are CD40-positive cell lines detected by flow cytometric
Tumor proliferation was measured with a methyl-thirotetrazol assay with values in control groups being 100%.

a: Significant differences between control and CD40L groups (grey) on Calu3 and Sq-1 (p<0.05).

b: Significant differences between IFN-γ (stripe) and CD40L+ IFN-γ groups (black) in CD40-positive cell lines (p<0.05).

In CD40-positive cell lines other than NCI-460, a significant decrease was observed in the number of CD40L-pulsed cells entering the S-phase (grey), whereas the population of the G0/G1-phase (stripe) was increased by CD40L pulsing. (The ungated cell population was excluded)

No significant changes were observed in CD40-negative cell lines.
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analysis.

Significant inhibition of tumor cell growth after CD40L stimulation was observed on Calu3 (78.0 ± 1.2%) and Sq-1 (89.3 ± 0.1%) cell lines compared with untreated cells (p<0.05). The NCI-460 cell line showed 15 to 20% inhibition in this cell proliferative assay at a CD40L concentration of 10μg/ml (data not shown). Also, significant inhibition was recognized after CD40L coincubation with INF-γ stimulation in all CD40-positive cell lines compared with treatment with INF-γ alone (p<0.05; Fig. 1).

CD40L stimulation was associated with an increase in the proportion of cells in the G0/G1 phase of the cell cycle and a decrease in the proportion of cells entering the S-phase of the cell cycle in all CD40-positive lung cancer cell lines. However, no changes in the proportion of CD40-negative tumor cells in the phases of the cell cycle were observed after stimulation with CD40L (Fig. 2). Stimulation of cancer cells with CD40L did not induce apoptosis or necrosis in a dual-channel flow cytometry assay with cell stained with annexin V and propidium iodide (data not shown).

Subtraction PCR analysis identified multiple genes associated with tumor suppressor activity, including Radixin (Calu3) and Moesin (NCI-460), and also identified cell cycle regulator genes, including SFRSS (Calu3) and MOV34 (NCI-460).

Real-time PCR analysis showed a 5-fold up-regulation of Radixin mRNA copy number (normalized by GAPDH mRNA copy number) after CD40L pulsing on Calu3.

Conclusion

CD40L inhibited the proliferation of CD40-positive lung cancer cell lines was through G0/G1 cell cycle arrest rather than an increase in apoptosis.

Coincubation with CD40L and INF-γ resulted in a greater inhibition of cell proliferation in CD40-positive cell lines.

Subtraction PCR analysis identified important genes expressed by CD40L pulsing which were related to tumor-suppressor genes and cell cycle regulator genes.

Up-regulation of Radixin was verified with a real-time PCR analysis of Calu3.

In the future, an in vivo study should be performed to examine the effectiveness of CD40-CDD40L stimulation as a new adjuvant therapy after surgery for lung cancer.