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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/m*l*) and/or INF-γ (100U/m*l*) 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%.

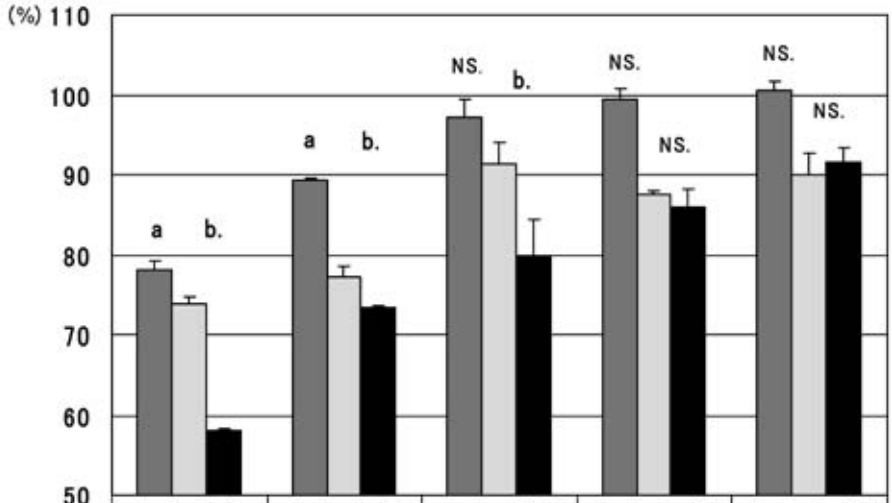
A 5-bromo-2'-deoxyuridine (Brd-U) flow cytometry method was used for cell cycle analysis after 72 hours of incubation with or without CD40L (1μg/m*l*).

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.

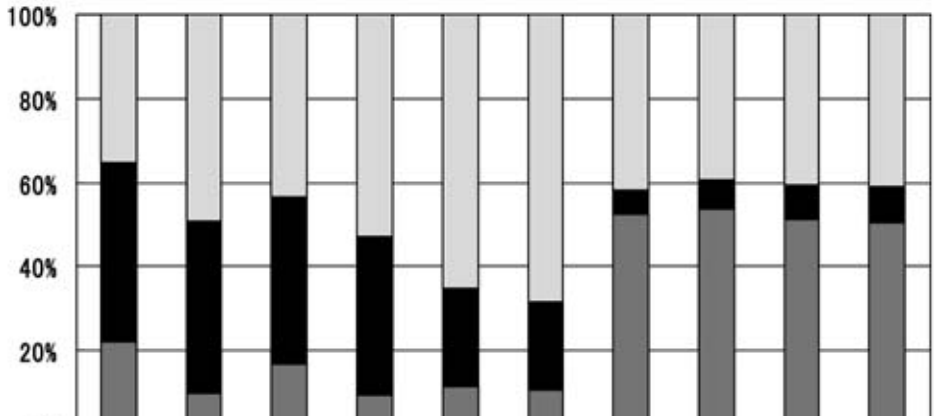
Results

Three of the five cell lines, Calu3, NCI-460, and Sq-1, are CD40-positive cell lines detected by flow cytometric



	Calu3(+)	Sq-1(+)	NCI460(+)	NCI1155(-)	NCI1355(-)
CD40L	78.0	89.4	97.1	99.5	100.7
IFN	73.8	77.3	91.3	87.6	90.0
CD40L+IFN	58.1	73.3	79.6	86.0	91.6

Fig. 1 Tumor proliferation was measured with a methyl-thiotetrazol 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 ($p<0.05$).



	Calu3	Calu3 +CD40L	Sq-1	Sq-1 +CD40L	NCI460	NCI460 +CD40L	NCI1155	NCI1155 +CD40L	NCI1355	NCI1355 +CD40L
G0/G1	34.0	46.6	40.6	49.5	63.1	67.1	40.1	37.8	39.5	40.0
G2+M	41.0	39.1	36.9	35.0	22.7	20.2	5.5	6.7	8.3	8.4
S	21.2	9.2	15.6	8.8	11.2	10.4	50.3	51.8	50.0	49.2

Fig. 2 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.

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/m*l* (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 SFRS5 (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.