Tuberculosis and Respiratory Diseases 결핵 및 호흡기질환, Vol. 49, No. 2, Aug, 2000

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TIMP-2 Gene Transfer Via Adenovirus Inhibits the Invasion of Lung Cancer Cell

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= 국문초록 =

TIMP-2 유전자 재조합 아데노바이러스의 폐암세포 침윤 억제 효과

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오연목, 이재호, 유철규, 정희순, 김영환, 한성구, 심영수, 이춘택

연구 배경 : 폐암은 진단 당시 이미 국소 침윤이나 원격 전이가 된 경우가 많고 이에 대한 적절한 치료법이 없기 때문에 예후가 불량하다. TIMP(tissue inhibitor of metalloproteinase)는 암세포의 침윤 및 전이에 중요한 역할을 하는 metalloproteinase를 억제하는 물질로서 생체 내에 존재하는 전이 억제 물질이다. 본 연구는 아데노바이러스를 이용한 TIMP 유전자 치료법을 개발하여 폐암의 치료에 응용하고자 하였다.

방 법 : 폐암세포는 침윤 및 전이 능력이 큰 Calu-6를 사용하였다. TIMP-2 유전자를 pACCMVpLpA에 subcloning 한 후 pJM17과 함께 293 cell에 cotransfection 한 후 homologous recombination을 이용하여 Ad-TIMP-2를 제작하였다. Ad-TIMP-2를 Calu-6 cell에 이입하여 TIMP-2 protein이 생산되는지를 TIMP-2 ELISA를 이용하여 확인하였고 TIMP-2의 생물학적 활성은 zymography로 확인하였다. Soft agar clonogenic assay로 종양형성능을 평가하였다. Ad-TIMP-2로 처리한 calu-6를 6주간 soft agar에서 키운 후 육안으로 보이는 colony 수를 측정하였다. Matrigel을 이용하여 invasion assay를 시행하여 calu-6의 침윤 능력의 변화를 평가하였다.

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결 과 : TIMP-2 ELISA 결과, 모세포 calu-6와 Ad-β-gal 이입 calu-6 그리고 Ad-TIMP-2 이입 calu-6는 각각 0.44, 0.43, 20.7 μg/10^6 cells/72hrs의 TIMP-2를 생산하였다. Zymography 결과 Ad-TIMP-2에 의해 생산된 TIMP-2는 matrix metalloproteinase-2의 gelatin 분해 효과를 억제하여 생물학적 활성을 확인할 수 있었다. Soft agar clonogenic assay 결과, 모세포인 calu-6는 453±53개, Ad-β-gal과 Ad-TIMP-2 이입된 calu-6는 각각 332±35, 280±45개의 colony가 형성되어 유의한 감소를 보이지 못했다. Invasion assay 로 모세포 calu-6에 대한 침윤율을 평가한 결과, Ad-β-gal과 Ad-TIMP-2가 이입된 calu-6(10moi)는 각각 71±8.9%, 12±8.4%의 침윤율을 보였으며 β-gal군에 비해 TIMP-2군이 유의하게 침윤율이 낮았다.
결 론 : Ad-TIMP-2는 폐암 세포의 종양형성능을 억제하지 못하였으나 침윤은 억제하여 TIMP-2가 폐암 유전자 요법에 이용될 가능성을 제시해 주었다. (Tuberculosis and Respiratory Diseases 2000, 49 : 189-197)

Key words : TIMP-2, Gene transfer, Lung cancer, Invasion.

Introduction

One of the reasons why the majority of patients with lung cancer do not have long term survival is early invasion and/or metastasis against which current modalities of treatment are usually ineffective[1]. For invasion, the initial step of metastasis, cancer cells should proteolyse basement membrane and matrix[2]. A group of the proteolytic enzymes involving the invasion of cancer cells are metalloproteinases, against which tissue inhibitor of metalloproteinase (TIMP) is known to act in vivo[3]. Four types of TIMP, TIMP-1, -2, -3, and -4 were discovered[4-8]. TIMP-2 out of them is known to inhibit the action of inactive as well as active form of matrix metalloproteinase-2 (MMP-2, 72kd type Ⅳ collagenase, gelatinase A) which activity was correlated with the metastatic potential of lung cancer cell lines[9]. TIMP-2 inhibits the invasion of human fibrosarcoma cell in vitro[10]. Also TIMP-2 gene transfer into rat embryo fibroblast transformed with *ras* inhibits it to metastasize to lung in nude mice[11].

We tried to answer the question whether TIMP-2 gene transfer via adenovirus inhibits the invasion of human lung cancer cell *in vitro* for the future purpose for TIMP-2 gene therapy against lung cancer.

Materials and Methods

1. Cells

Calu-6, a human lung cancer cell line, was obtained from Korean Cell Line Bank, Seoul, Korea. Cells were cultured in RPMI 1640 with 8% of fetal bovine serum.

2. Construction of recombinant Ad-TIMP-2

Using modification of Graham's method we constructed recombinant Ad-TIMP-2[12]. The cDNA of TIMP-2 (a gift of Dr. WG Stetler-Stevenson, National Cancer Institute, National Institutes of Health) was subcloned into pACCMVpLpA (a gift of Dr. Robert Gerard, University of Texas Southwestern Medical Center) to make pACCMV-TIMP-2. To verify the structure of recombinant pACCMV-TIMP-2, it was sequenced around 5'-end and 3'-end of inserted TIMP-2 cDNA using ABI 373A DNA Sequencer (Perkin-Elmer, Cambridge, UK) with primers of both ends, GCAGACCTCGTTTAGTGA-

ACC and GTCCAATTA-TGTCACACCACAG. The resulting pACCMV-TIMP-2 and pJM17 (also a gift of Dr. Gerard) were cotransfected into 293 cell by standard calcium phosphate coprecipitation method. Adenovirus-TIMP-2 (Ad-TIMP-2) was generated by homologous recombination. A recombinant adenovirus expressing the lacZ gene under the control of CMV promotor was used as a control virus (Ad-β-gal).

3. Expression of TIMP-2 gene from Ad-TIMP-2 transduced lung cancer cell

Calu-6 was transduced with Ad-TIMP-2 of 30moi and was confirmed to produce biologically active TIMP-2 by ELISA (Tissue inhibitor of metalloproteinases-2, human, ELISA system, Amersham, UK) and reverse zymography. Reverse zymography was done as follows[10]. Sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis was performed using 12%(w/v) acrylamide gel into which 0.1% (w/v) gelatin was incorporated prior to polymerization. 10 μl of conditioned media obtained 72 hours after transduction of calu-6 with Ad-TIMP-2 or Ad-β-gal, were electrophoresed at 4℃ without heat treatment. As a positive control, 0.6ng of TIMP-2 was used during gel electrophoresis. After electrophoresis, gel was incubated in 2.5 % (v/v) Triton X-100 for 1 hour twice to remove SDS. Being treated with 0.5 μg of matrix matelloproteinase-2 (MMP-2, Oncogene, Cambridge, MA), gel was incubated at 37℃ in 50mM Tris-200mM NaCl-10mM $CaCl_2$, pH 7.5 for 24 hours to allow proteolysis of gelatin. Then gel was stained with 0.25% (w/v) Coomassie brilliant blue and destained in methanol : acetic acid : water (50 : 10 : 40). A clear zone indicates gelatinolytic activity, while a dark zone indicates an inhibitor of gelatinase.

4. Soft agar clonogenicity

Anchorage-independent growth was assessed by soft agar clonogenicity assay. Briefly, calu-6 was transduced with Ad-TIMP-2 or Ad-β-gal (30moi) and then was detached and plated in 0.2% agarose with a 1% underlay (5×10^3 cells/plate). Colonies were counted after 6 weeks.

5. Invasion assay

To quantify invasion, in vitro invasion assay was done using two chamber well (Transwell, Costar, Cambridge, MA) divided by in-between matrigel (Collaborative Research, Waltham, MA) and filter. Calu-6 cells, parental and transduced with Ad-TIMP-2 or Ad-β-gal (10 or 30moi), were plated on Transwell inserts (6.5mm) containing polycarbonate filters with 8 μm pores. The upper surface of the filters was coated with 100 μl matrigel (1 : 20 dilution) which was dried overnight to allow matrigel to form gel. 8×10^4 calu-6 cells, parental and transduced, were plated on the filter in RPMI1640 with 8% of fetal bovine serum. 500 μl of serum free RPMI1640 containing 10 μg/ml (w/v) fiibronectin (Bacton Dickinson, Bedford, MA) as a chemo-attractant was treated on each lower well. Cells that invaded through matrigel and filter were counted after 72-hour incubation.

Results

1. DNA sequencing

As the result of DNA sequencing, cDNA of TIMP-2 was confirmed to be inserted into pACCMVpLpA correctly (Fig. 1).

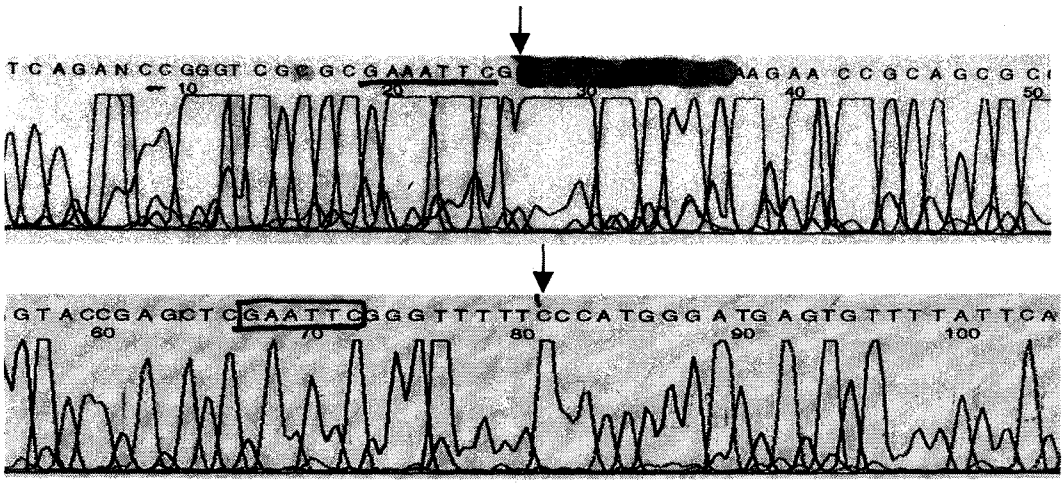

Fig. 1. Sequencing of pAC CMV-TIMP-2 confirmed the correct insertion of TIMP-2 at EcoRI restriction(GAATTC) of pUC19 polylinker of pAC CMV pLpA. The upper one is the sequencing around 5'-end of inserted TIMP-2 cDNA and the lower one is around 3'-end. Notice arrows are the ends of TIMP-2 cDNA.

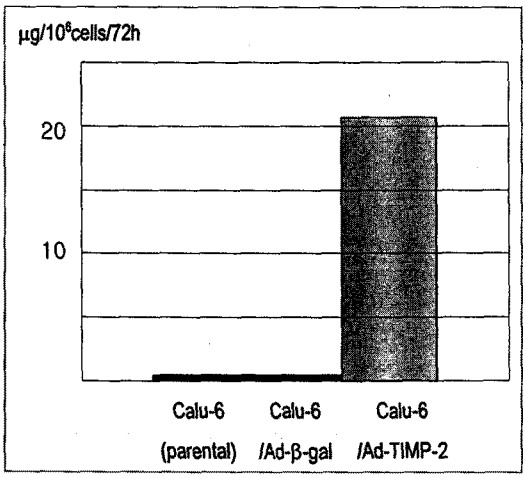

Fig. 2. By TIMP-2 ELISA, parental calu-6, calu-6/Ad-β-gal, and calu-6/Ad-TIMP-2 produced 0.44, 0.43, and 20.7 μg/10⁶cells /72hrs of TIMP-2 respectively.

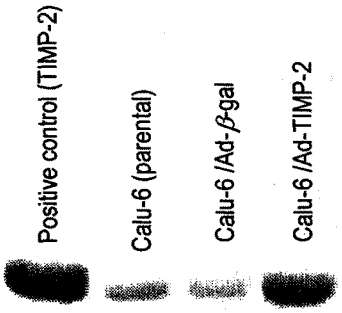

Fig. 3. Reverse zymography showed that the produced TIMP-2 inhibits the gelatinase activity of matrix metalloproteinase-2. A clear zone indicates gelatinolytic activity, while a dark zone indicates an inhibitor of gelatinase. Conditioned media from calu-6 / Ad-TIMP-2 (last band) had inhibitory activity against gelatinase activity comparable to that of the purified TIMP-2 enzyme (first band).

2. TIMP-2 ELISA

Parental calu-6, calu-6 transduced with Ad-β-gal (calu-6/Ad-β-gal), and calu-6 transduced with Ad-TIMP-2 (calu-6/Ad-TIMP-2) produced 0.44, 0.43, and 20.7 μg/10^6 cells/72hrs of TIMP-2 respectively (Fig. 2). Calu-6/Ad-TIMP-2 produced TIMP-2 more than 50 times than parental and calu-6/Ad-β-gal.

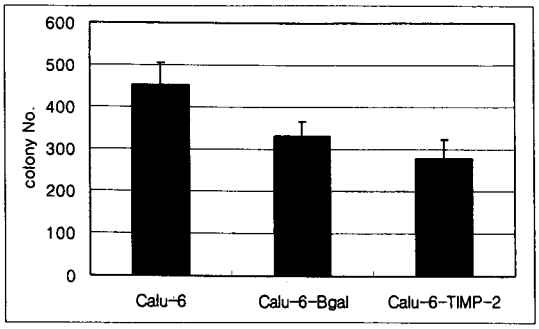

Fig. 4. By soft agar clonogenicity assay, the number of colonies was 453±53, 332±35, and 280±45 for parental calu-6, calu-6/Ad-β-gal(30moi), and calu-6 / Ad-TIMP-2(30moi) respectively. Anchorage-independent growth was not different between calu-6 / Ad-β-gal, and calu-6 / Ad-TIMP-2. Error bars mean the standard deviation of the three separate experiments.

3. Reverse zymography

The produced TIMP-2 inhibited the gelatinase activity of matrix metalloproteinase-2. In Fig. 3, a clear zone indicates gelatinolytic activity, while a dark zone indicates an inhibitor of gelatinase. Conditioned media from calu-6 / Ad-TIMP-2 (last band) had inhibitory activity against gelatinase activity comparable to that of the purified TIMP-2 enzyme (first band). This finding means TMIP-2 produced by calu/Ad-TIMP-2 is biologically active.

4. Soft agar clonogenicity

The numbers of colonies by soft agar clonogenicity assay were 453±53, 332±35, and 280±45 for parental calu-6, calu-6 / Ad-β-gal, and calu-6 / Ad-TIMP-2 respectively (Fig. 4). Anchorage-independent growth was not different between calu-6 transduced with Ad-β-gal and calu-6 transduced with Ad-TIMP-2.

5. Invasion assay

The relative invasions of calu-6 / Ad-β-gal and calu-6 / Ad-TIMP-2 to parental cell were 71±8.9% vs. 12±8.4% at 10moi and 31±6.5% vs. 10±6.4% at 30moi, respectively (Fig. 5). Relative invasions of calu-6-Ad-TIMP-2 were much less than those of calu-6-Ad-β-gal ($p<0.05$: unpaired t test, SPSS).

Discussion

Lung cancer is notorious for its poor prognosis because of early invasion and metastasis, which current treatment modalities cannot counteract efficiently. Therefore the inhibition of invasion and / or metastasis in lung cancer patients might be one of potential strategies to improve their prognosis. The invasion, the initial step of metastasis, is active and complicated process in which many proteolytic enzymes especially matrix metalloproteinases are necessary to dissolve extracellular matrix. There are natural inhibitors of metalloproteinases such as TIMP-1, -2, -3 and -4. If protease activity overwhelms inhibitor activity, cancer cells could invade and metastasize[2]. This hypothesis is supported by the following studies. Librach showed indirectly by demonstrating antibody against MMP-9 inhibits cytotrophoblast invasion that matrix metalloproteinase-9 (92-kD type IV collagenase) mediates invasion of human cytotrophoblasts[13]. Clarke reported that type IV collagenase expression in metastasizing adenocarcinoma of the lung is much

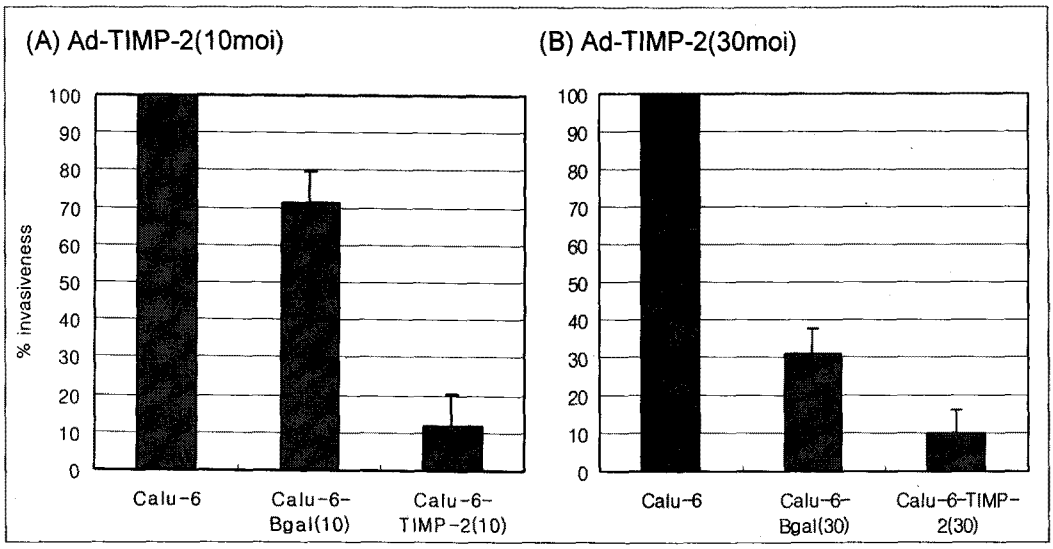

Fig. 5. Invasion assay by two-chamber matrigel assay. (A) The relative invasions of transduced calu-6 to parental cell were 71±8.9% (calu-6 / Ad-β-gal, 10moi), and 12±8.4% (calu-6 / Ad-TIMP-2, 10moi) respectively. (B) The relative invasions of transduced calu-6 to parental cell were 31±6.5% (calu-6 / Ad-β-gal, 30moi), and 10±6.4% (calu-6 / Ad-TIMP-2, 30moi) respectively. Relative invasions of calu-6 / Ad-TIMP-2 in both moi's were much less than calu-6 / Ad-β-gal ($p<0.05$). Error bars mean the standard deviation of six separate experiments.

higher than that in non-metastasizing one[14]. Zucker confirmed calu-6 and A549 that secreted the highest levels of MMP-2 out of several lung cancer cell lines had highest metastatic, invasive and tumorigenic potential[9]. Nawrocki found out that mRNA levels of MMP and its expression frequencies in patients with bronchopulmonary carcinomas increased progressively with malignant phenotype, lack of differentiation and TNM staging of tumors[15].

To inhibit invasion and metastasis of cancer cell, the augmentation of the inhibitor activity might be a possible method. In order to confirm that the augmentation of the inhibitor activity could reduce invasion and metastasis of lung cancer cells, we performed this study. TIMP-2 activity was augmented by adenovirus vector in our study. And calu-6, one of the most invasive and metastatic lung cancer cells was transduced with recombinant Ad-TIMP-2[9,16].

The augmentation of TIMP-2 activity already had been shown to reduce invasion and metastasis of cancer cells other than in lung cancer. Albini reported that TIMP-2 inhibits *in vitro* invasion of human fibrosarcoma cells[10]. Ahonen showed adenovirus-mediated gene delivery of TIMP-2 inhibits invasion in melanoma cells[17]. Declerk reported that rat embryo cells transfected with TIMP-2 using calcium phosphate method showed reduced invasion and metastasis in nude mice, and showed that TIMP-2 augmentation does not only inhibit invasion of cancer cells but also inhibit tumorigenicity[11]. In our study TIMP-2 gene transfer did not inhibit

tumorigenicity *in vitro* but did inhibit invasion *in vitro*. The difference of tumorigenicity between our study and others might be explained as that between *in vitro* and *in vivo*. *In vivo* tumor growth necessarily needs angiogenesis that also requires metalloproteinase. TIMP-2 might inhibit *in vivo* tumor growth by inhibiting angiogenesis. However, our study was not performed *in vivo* but *in vitro* and did not show any difference of tumorigenicity.

The invasion assay in our study revealed that Ad-TIMP-2 transduction reduced the invasiveness of calu-6. In lower moi(10moi), the difference was greater [71%(Ad-β-gal)/12%(Ad-TIMP-2)] than that in higher moi(30moi) [31%(Ad-β-gal)/10%(Ad-TIMP-2)]. The decrease in invasiveness by Ad-β-gal(30moi) might be related with the reduction of tumor cell growth by adenovirus itself, not the reduction of invasiveness. Actually, the result of invasion assay reflects the rate of invasion and rate of growth of cancer cell.

In conclusion, TIMP-2 gene transfer via adenoviral vector can reduce the invasiveness of high invasive lung cancer cell line. This could be a valuable finding for designing of combination therapeutic modality in cancer. Future study will focus on the combination with other gene therapy or conventional treatments such as chemotherapy and radiation therapy.

Abstract

Background : Tissue inhibitor of metalloproteinase is a natural inhibitor that counteracts proteolytic enzymes essential to the invasion of cancer cell. Whether or not TIMP-2 gene transfer via adenovirus could inhibit the invasion of lung cancer cell *in vitro* was evaluated for the future purpose of gene therapy against lung cancer.

Methods : Recombinant adenovirus-TIMP-2(Ad-TIMP-2) was generated by homologous recombination after pACCMV-TIMP-2 and pJM17 were cotransfected into 293 cell by standard calcium phosphate coprecipitate method. Calu-6, one of the most invasive lung cancer cells, was transduced with Ad-TIMP-2 or Ad-β-gal. Anchorage-independent growth and invasiveness were assessed by soft agar clonogenicity assay and invasion assay using two-chamber, well divided by matrigel.

Results : Ad-TIMP-2 transduced calu-6 cells produced biologically active TIMP-2 more than 50 times more than parental calu-6. TIMP-2 gene transfer did not suppress the *in vitro* tumorgenicity. However, two chamber well assay revealed that Ad-TIMP-2 transduction reduced the invasiveness of calu-6 efficiently (12 % compared with parental cell) even at low 10moi.

Conclusion : Even though TIMP-2 gene transfer did not inhibit *in vitro* tumorigenicity, it did inhibit invasion of lung cancer cell *in vitro*. The inhibition of invasion by Ad-TIMP-2 may be a useful strategy for the treatment of lung cancer.

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