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CONSTRUCTION AND EXPRESSION OF THE REPLICATION-DEFICIENT ADENONIRUS VECTOR OF HUMAN GM-CSF*

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The full-length cDNA encoding human Granulocyte-macrophage colony-stimulating factor (GM-CSF) was cloned by RT-PCR, placed under the control of CMV promoter, and inserted into adenovirus vector of E1-substitution type, pAx1cw. Subsequently, the cassette cosmid was cotransfected into 293 cells together with EcoT22I-digested Ad5-TPC, and the replication-deficient recombinant adenoviruses(Ad) of human GM-CSF were generated efficiently by homologous recombination, with the titers of 1.51×10^9pfu/ml. 48 hours after infection with prepared human GM-CSF recombinant adenoviruses in vitro, HeLa cells and primary human skin fibroblasts expressed high levels of human GM-CSF (80~400ng/ 10^6cells/24hr). These suggest that the recombinant Ad of human GM-CSF prepared by COS/TPC method is effective in mediating GM-CSF gene transfer and might be used in cancer gene therapy.

Key words: Granulocyte-macrophage colony-stimulating factor, Adenovirus vector, Gene transfer, Gene expression

Cytokine immunogene therapy is one of major approaches to cancer gene therapy, the main advantage of which is the potential to generate potent systemic antitumor responses.[1] GM-CSF capable of upregulating immune responses is one of the most promising cytokines in cancer immunogene therapy.[2] Autologous tumor vaccines transfected with human GM-CSF retrovirus vector have been approved in U.S.A. to enter clinical trials to treat the patients with metastatic melanoma, renal carcinoma or prostate carcinoma[1]. Adenovirus vector capable of mediating gene transfer at high efficiency regardless of the mitotic status of the cell attracts great attention in gene therapy. In this study, we constructed the recombinant adenovirus of human GM-CSF by COS/TPC method[3] and determined its *in vitro* expression.

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MATERIALS AND METHODS

Vectors

The charomid vector pAx1cw (gift from Dr. I. Saito , the University of Tokyo, Japan) is used for constructing recombinant adenovirus of E1-substitution type, which bears an Ad5 genome spanning 0-99.3 map units(mu) with deletions of E1(mu 1.3-9.3) and E3(mu79.6-84.8). The cloning sites are ClaI and SwaI. The eukaryotic expression vector pCIcc was derived from pCI (Promega), by inactivation of BglII site and insertion a ClaI site upstream of CMV promoter. Therefore, pCIcc vector contains two ClaI sites locating at the upstream of CMV promoter and the downstream of SV40 polyA respectively. Exogenous cDNA can be inserted into pCIcc vector, and the resultant ClaI fragment of expression cassette can be cloned into pAx1cw to construct its recombinant Ad vector. The cloning vector pBluescipt-SKII was from Stratagene.

Bacterials and Cell Lines

293 is a human embryonic kidney cell line transformed with Ad5 E1A and E1B genes and supports propagation of E1-deleted recombinant Ad. HeLa is a human cervix epitheloid carcinoma cell line. The above cell lines and primary human skin fibroblasts were cultured in DMEM (Gibco) supplemented with 10% FCS (Hyclone). DH5α and MC1061 bacterial strains were used in molecular cloning.

Cloning of Human GM-CSF cDNA

The cDNA encoding human GM-CSF was cloned from activated human T lymphocytes by RT-PCR. Human PBMCs were prepared from whole blood by gradient separation using Ficoll-Paque and subjected to the cellect^{TM} Human T Cell kit(Biotex Laboratories Inc. Alberta, Canada) for human T cell enrichment. The enriched T cells were stimulated in vitro with Con A (10μg/ml, Sigma) for 24h, subsequently from which the total cellular RNA was extracted with guanidine thiocyanate. 5μg total RNA was mixed with 1μl (0.5μg) oligo-dT and 1μl 10mM dNTP, incubated at 70 ℃ for 10 min, subsequently added AMV reverse transcriptase, and incubated at 42 ℃ for 50min,then terminated at 70 ℃ for 10 min. 2μl reverse transcripts were used as templates for amplifying human GM-CSF. The upstream primer used for human GM-CSF was 5'CGGGAATT-CTAGACCACCATGTGGCTGCAGAGCCTG3', and the downstream primer 5'CGGGGATCCTCACT-CCTGGACTGGCTCCC3', with the expected products in size of 456bp. PCR was performed in the volume of 50μl containing 30pmol upstream and downstream primers each, 1.5mM $MgCl_2$, and 2U Taq DNA polymerase. The parameters of PCR amplification were 96 ℃ 1min, 55 ℃ 1min, 72 ℃ 2min. After 20 cycles of amplification, the PCR products were subjected to electrophoresis on 2% agarose gel. The PCR products were digested with EcoRI and BamHI, then cloned directionally into pBluescript-SKII vector. The recombinant vector pSK-hGM was subjected to automatic sequencing using T3 and KS primer.

Insertion of Human GM-CSF Expression Cassette Into pAx1cw

The XbaI fragment of human GM-CSF from pSK-hGM was inserted into pCIcc vector at the XbaI site to construct the recombinant vector pCI-hGM, which bears an expression cassette consisting of CMV promoter, human GM-CSF cDNA and SV40 polyA. Then, the human GM-CSF expression cassette was excised with ClaI, purified by gel electrophoresis, and mixed with ClaI-linearized and CIAP-treated pAx1cw cassette DNA. After overnight ligation with T4 DNA ligase (NEB) at 14 ℃, the ligate was in vitro packaged by Gigapack XL (Stratagene) and then transduced into *E.coli* DH5α. The cosmid DNA of positive clone (pAx1cw.ClhGM) was largely prepared and purified through a buoyant CsCl density gradient by centrifugation in a Vti65 rotor (Beckman) for 16hr at 55,000rpm.

Preparation of EcoT22I-Digested Ad5 DNA-TPC

Ad5-dlX with deletion of E1 and E3 was used as parent virus for construction of recombinant Ad. The virions were prepared from Ad5-dlX-infected 293 cells through a buoyant CsCl density gradient and lysed by addition of an equal volume of 8M guanidine hydrochloride to release Ad5 DNA-TPC. The DNA-TPC was purified through a buoyant density gradient of 2.8M CsCl/4M guanidine hydrochloride by centrifugation in a Vti65 rotor for 16hr at 55,000rpm and dialyzed. The DNA-TPC was then digested with EcoT22I and subjected to gel filtration through a Sephadex G-50 spin column. The digested DNA-TPC was aliquoted and stored at −80 ℃.

Generation of Recombinant Adenoviruses of Human GM-CSF

40μg of cassette cosmid bearing human GM-CSF(pAx1cw.ClhGM) was cotransfected together with 0.5μg EcoT22I-digested Ad5 DNA-TPC into 293 cells in a 10-cm dish by calcium phosphate protocol. The desired recombinant Ad was generated by homologous recombination. One day later, the transfected 293 cells were spread in three 96-well plates at a 10-fold serial dilution mixed with untransfected 293 cells. After 10~15 days of culture, the virus clones were isolated and propagated further to assess restriction analysis. The recombinant Ad was titered using 293 cells as target cells.

In Vitro Expression of Human GM-CSF Recombinant Adenoviruses

The HeLa cells or primary human skin fibroblasts growing logarithmically were washed twice with HBSS buffer solution, and seeded with LacZ or human GM-CSF recombinant Ad at different MOIs (Multiplicity of Infection). 1 hour later, the Ad.solution was discarded and replaced with fresh DMEM complete medium. After 48 hr of culture at 37 ℃, the culture supernatants were harvested and measured for human GM-CSF with its capture ELISA kit(R&D).

RESULTS

Cloning of Human GM-CSF cDNA

The human GM-CSF cDNA with the expected size was amplified from the activated human T cells by RT-PCR(Figure 1) and inserted directionally at EcoRI and BamHI sites of pBluescript-SKII. The cloned human GM-CSF cDNA was confirmed through sequencing, and consistent with that previously reported.[4]

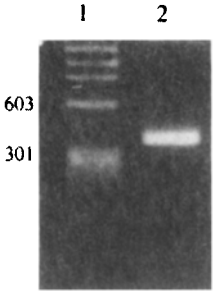

Fig. 1. Electrophoretic analysis for human GM-CSF RT-PCR products.

lane 1. ϕx174/HaeIII DNA marker

lane 2. human GM-CSF RT-PCR products

Construction of Recombinant Adenoviruses of Human GM-CSF

The human GM-CSF expression cassette was inserted into cassette cosmid pAx1cw at ClaI site. After in vitro package and plating the transduced E.coli DH5α, numerous colonies were obtained, the majority of which contained the desired insert. To generate recombinant Ad bearing human GM-CSF, the cassette cosmid bearing human GM-CSF was transfected into 293 cells together with EcoT22I-digested Ad5 DNA-TPC. EcoT22I cuts the E1-deleted Ad5 genome at four sites, all within the left one-third of the genome, thereby mimizing the potential of regeneration of intact parent virus. As shown in Figure 2, the desired recombinant Ad was generated by homologous recombination in 293 cells which occurs between the homologous regions of DNA-TPC and the cosmid cassette from the rightmost EcoT22I site to the right end of the Ad5 genome(23.2kb in length). By the second homologous recombination, the cosmid cassette sequence at the left end of Ad5 DNA are removed and replaced with the homologous inverted terminal repeat(ITR) sequence at the left end of DNA-TPC. 86 virus clones were obtained, 8 clones of which were propagated further for restrictive analysis of viral genome. As shown by Fig.3, all these 8 clones were positive and identified as the desired clones. The titer of the propagated 3rd recombinant Ad clone was 1.51×10^9 pfu/ml. This indicates COS/TPC recombination is an efficient and reliable method to generate recombinant Ad.

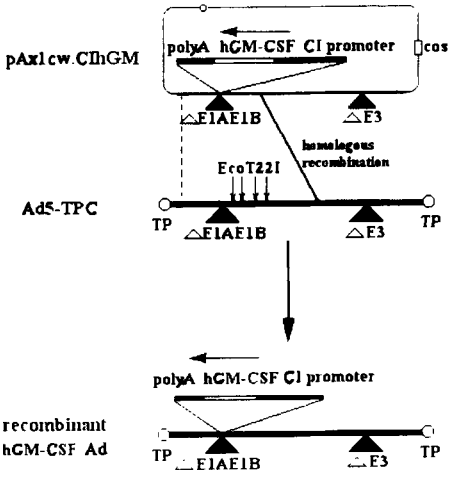

Fig. 2. Skematic demonstration for the generation of human GM-CSF recombinant adenovirus by COS/TPC homologous recombination.

In Vitro Expression of the Human GM-CSF Recombinant Adenoviruses

After infection with the LacZ recombinant Ad, HeLa cells and primary human skin fibroblasts were subjected to X-Gal staining. The results showed that

the gene transfer efficiency was higher than 95% at the MOI of 100. 48hr after infection with the recombinant Ad bearing human GM-CSF, these two kinds of cells expressed high levels of human GM-CSF (80~400ng/10^6cells/24hr), as demonstrated in Table 1. As the MOIs increased, the expression of human GM-CSF increased significantly. There is no detectable human GM-CSF in the supernatants from the untransfected cells and mock controls. This suggests that the prepared recombinant Ad of human GM-CSF can mediate human GM-CSF expression in vitro efficiently.

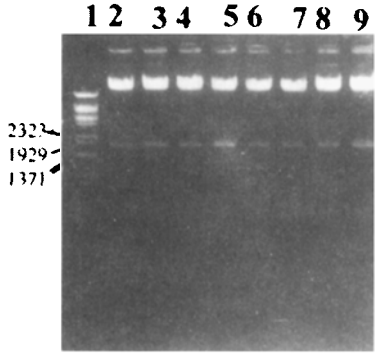

Fig. 3. Digestive analysis for the genomic DNA from the 293 cell clones generating recombinant human GM-CSF adenoviruses.

lane 1. λ/BstE II DNA marker

lane 2–8. ClaI digests of human GM-CSF adenovirus clones

Table1. The human GM-CSF expression by the cells infected with recombinant adenovirus of human GM-CSF

Infected cells	Human GM-CSF levels at different MOIs (ng/10^6 cells/24h)			
	0	50	100	200
HeLa cells	0	86± 6	136± 14	375± 29
Skin fibroblasts	0	105± 9	162± 15	394± 41

DISCUSSION

The tumor vaccines transduced with GM-CSF gene have been demonstrated to be capable of inducing long-lasting, potent, and specific antitumor immunity in murine tumor models. The antitumor effects of GM-CSF may be associated with its upregulating antigen presentation of antigen-presenting cells, e.g. dendritic cells. It has been shown that GM-CSF plays crucial role in the proliferation, differentiation, in vivo distribution, and functional regulation of dendritic cells.[5-6] As described in our previous research, GM-CSF gene transfection of dendritic cells could augment the antitumor responses induced by tumor antigen-pulsed dendritic cells.[7]

Retrovirual vector can mediate gene transfer efficiently into dividing cells and integrate into host genomic DNA, consequently express target gene stably. Up to now, it is used in the majority of approved clinical protocols of gene therapy[1]. However, there are several potential limitations for retroviral vector. First, retrovirus can only infect dividing cells, which limits its gene transfer efficiency, especially for in vivo delivery. Second, Retroviruses can be inactivated by human serum complement, although it was reported recently that the recombinant retroviruses produced by packaging cells of human origin resisted complement inactivation. Third, The currently achievable titers (10^7cfu/ml) of retroviruses are low compared with the need for treatment of the tumors with large volume. Fourth, it's difficult to prepare retroviruses in large scale. Fifth, its host range is limited. Although some modifications of retrovirus system may overcome the above limitations, more versatile vectors are attractive for successful application of gene therapy. Attention has been focused on adenovirus vector, since adonovirus can infect a variety of cell types with high-level transduction regardless of the mitotic status of the cells. The adenovirus genome is a 36-kb double-stranded NDA tagged with a 55-kD terminal peptide(TP) at both ends[8]. When transfected to permissive cells the adenovirus genome tagged with TP produce 100-fold more viral plaques than the proteinase-treated counterpart. COS/TPC was shown to be a efficient method to generate recombinant adenovirus, in which cosmid cassette bearing adenovirus genome is recombined homologously with Ad DNA-TP complex (DNA-TPC).

The recombinant adenovirus of human GM-CSF generated in this study is effective in mediating human GM-CSF gene transfer and expression. It might be applied in clinical trials in the future. For example it can be used to transfect tumor cells to prepare tumor vaccines, or transfect antigen-presenting cells, such as dendritic cells or macrophages. It can also be injected into tumor mass to directly deliver human GM-CSF

gene *in vivo*.

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