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CONSTRUCTION OF THE DICISTRONIC ADENOVIRUS VECTOR EXPRESSING BIOACTIVE HUMAN INTERLEUKIN-12*

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The full-length cDNA encoding the subunits p40 and p35 of human interleukin-12(hIL-12) were cloned separately by RT-PCR, linked together by internal ribosomal entry site (IRES) of encephalomyocarditis virus which initiates cap-independent translation to form a dicistronic gene fragment. The dicistronic fragment was placed between the cytomegalovirus (CMV) promoter and SV40 polyA signal to form a dicistronic expression cassette. Subsequently, the dicistronic expression cassette was inserted into E1 region of Ad5 genome in cosmid vector pAx1cw of E1-substitution type. By homologous recombination with EcoT22I-digested Ad5 DNA-TPC in 293 cells, the replication-deficient recombinant adenoviruses of hIL-12 were generated efficiently. After infected with hIL-12 recombinant adenoviruses in vitro, 293 cells, human hepatocellular carcinoma cells HepG2, and primary human skin fibroblasts expressed and secreted hIL-12 at comparable levels (30~60ng/ 10^6cells/24hr), which could stimulate the proliferation and IFN-γ production of human lymphoblasts. These suggest that the dicistronic adenovirus vector of hIL-12 could effectively mediate the expression of bioactive hIL-12 and might be used in cancer gene therapy.

Key words: Interleukin-12, Dicistronic vector, Adenovirus vector, Internal ribosomal entry site, Gene expression.

Interleukin-12(IL-12) is a 75kD heterodimer composed of two disulfide-linked subunits in size of 35 and 40kD. Produced mainly by antigen-presenting cells such as macrophages, B cells and dendritic cells, IL-12 plays central role in the induction of Th1-mediated immunity.[1] Recent reports revealed the potential of IL-12 in the anti-infection immunotherapy and cancer immunotherapy. [2-4] It was also found that systemic and prolonged administration of IL-12 led to severe side-effects. An promising alternative of IL-12 delivery is to take advantages of viral vector.[5-6] Fibroblasts genetically engineered with hIL-12 retroviral vector have been approved in U.S.A. to enter clinical trial to treat melanoma patients.[7] Adenovirus vector has several advantages over retroviral vector, such as high gene transfer efficiency regardless of cellular mitotic status and *in vivo* efficient delivery of therapeutic gene. In this paper, we constructed the dicistronic adenovirus vector of hIL-12 using internal ribosomal entry site(IRES) of encephalomyocarditis virus (EMCV) which mediates initiation of cap-independent translation.[8]

MATERIALS AND METHODS

Reagents

The eukaryotic expression vector pCIcc and the adenovirus vector of E1-substitution type, pAx1cw, have been previously described. [9] Recombinant hIL-12 was purchased from Genzyme Co., and ^{3}H-TdR from Amersham, PDBu (phorbol-12,13-dibutyrate) from

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Sigma. The hIL-12 and human IFN-γ ELISA kits were obtained from R&D Co.

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 Ads. KB is a human epitheloid carcinoma cell line(ATCC CCL17), and HepG2 is a human hepatocellular carcinoma cell line(ATCC HB8065). The above cell lines and human primary skin fibroblasts were cultured in DMEM (Gibco) supplemented with 10% FCS (Hyclone). DH5α and MC1061 bacterial strains were used in molecular cloning.

cDNA Cloning of hIL-12 Subunits

KB cells were stimulated with PDBu(100nM) at 37 ℃ for 20h before extraction of total cellular RNA with guanidine thiocyanate. 5μg total RNA mixed with 0.5μg oligo-dT was incubated at 70 ℃ for 10 min, subsequently added AMV reverse transcriptase, and incubated at 42 ℃ for 50min, followed by termination at 70 ℃ for 10 min. 2μl reverse transcripts were used as templates for amplifying the p35 and p40 subunits of hIL-12 respectively. The upstream primer used for human p40 was 5'GCTCTAGACTGCCATGT-GTCACCAGCAGTTGGTC3', and the downstream primer 5'CGGGATCCCTAACTGCAGGGCACAG-ATGC3', with the expected products in size of 1003bp. The upstream primer for p35 is 5'GGGGTACCT-CTAGACTCAGCATGTGTCCAGCGCGCAGCCTC 3', and the downstream primer 5'CGTCCGGATC-CTTAGGAAGCATTCAGATAGCTC3', with the expected products of 676bp. PCR was performed in the volume of 50μl containing 30pmol upstream and downstream primer each, 1.5mM $MgCl_2$, and 2U Taq DNA polymerase (PE Co.). The parameters of PCR amplification were 96 ℃ 1min, 60 ℃ 1min, 72 ℃ 2min. After 25 cycles of amplification, the PCR products were subjected to electrophoresis on 1.5% agarose gel. The PCR products were digested with appropriate restrictive endonucleoenzyme, then inserted directionally into pBluescript-SKII vector. The recombinant vector pSK-h40 and pSK-h35 were subjected to automatic sequencing.

Construction of Dicistronic Expression Cassette of hIL-12

As demonstrated in Figure1, p35, IRES and p40 cDNA were cloned into pCIcc vector sequentially. Resultantly, IRES was placed between p40 and p35 to form dicistronic fragment which was under the control of CMV promoter and followed by SV40 polyA.

Generation of Recombinant Adenovirus of hIL-12

The ClaI fragment containing the dicistronic expression cassette was inserted into cosmid vector pAx1cw at the ClaI site, which followed by in vitro packaged by Gigapack XL (Stratagene). The cosmid DNA of positive clone (pAx1cw.CIh40i35) was largely prepared and purified through a buoyant CsCl density gradient by centrifugation in a Vti65 rotor (Beckman) for 16hr at 55,000rpm. The purified cassette cosmid bearing hIL-12 was cotransfected together with EcoT22I-digested Ad5 DNA-TPC into 293 cells as described previously.[9] The desired recombinant adenoviruses was generated by homologous recombination.[9]

***In vitro* Expression of hIL-12 Recombinant Adenoviruses.**

293 cells, HepG2 cells or primary human skin fibroblasts were infected with hIL-12 recombinant Ad at different MOIs (Multiplicity of Infection). 48hr after infection, their culture supernatants were harvested and subjected to hIL-12 detection with both ELISA immunoassay and bioassay. ELISA assay was performed according to the manufacturer's instructions.

Bioassay for hIL-12

PHA-stimulated human PBMCs were used to measure the bioactivity of hIL-12. Briefly, PBMCs separated from healthy adult donor by Ficoll-Paque (Pharmacia) density gradient were stimulated in vitro with PHA(10μg/ml, Sigma) at 37 ℃, 5%CO_2 for 72hr. Subsequently, 2×10^4 PHA-stimulated PBMCs were seeded into each well of 96-well culture plates(Nucon) and cocultured with hIL-12 standard or samples at different dilutions at 37 ℃ for 60hr. 12hr before the end of culture, 1μCi ^{3}H-TdR was added into each well. The cells were harvested for counting cpm with liquid scintillation counter, and the culture supernatants were subjected to IFN-γ measurement with human IFN-γ

ELISA kit.

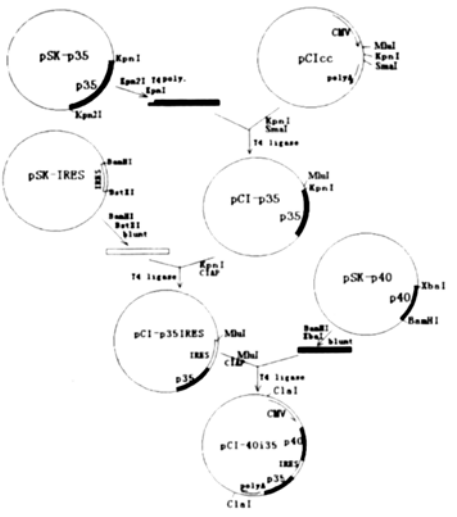

Fig. 1. Flow diagram of the construction of hIL-12 dicistronic expression cassette pCI-40i35.

RESULTS

cDNA Cloning of The Subunits of Hil-12

The p40 and p35 cDNA of hIL-12 were amplified separately from the activated KB cells by RT-PCR, the sizes of which are consistent with that predicted (Figure 2). After being cloned into pBluescript-SKII vector(Strategene), they were subjected to automatic sequencing, and the right clones with sequences consistent with that previously reported[10] were obtained.

Construction of Dicistronic Adenovirus Harboring hIL-12

First, p35, IRES and p40 cDNA were cloned into cassette pCIcc vector sequentially to form dicistronic expression cassette. In the resultant pCI-40i35 vector, IRES was located between p40 and p35 cDNA, and transcription of the fusion gene p40-IRES-p35 was directed by CMV promoter. The translation of p40 and p35 from a single transcript was directed by cap-dependent and cap-independent mechanisms respectively. Then, the fragment of dicistronic expression cassette was cloned into cosmid vector pAx1cw at ClaI site to construct dicistronic adenovirus vector pAx1cw. CIh40i35.

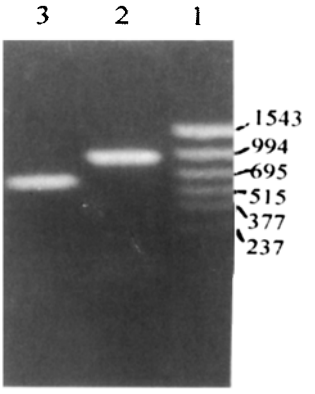

Fig. 2. Electrophoretic analysis for the RT-PCR products of hIL-12 subunits.

lane 1. PCR marker

lane 2. human p40 RT-PCR products

lane 3. human p35 RT-PCR products

After transfection of pAx1cw.CIh40i35 together with EcoT22I-digested Ad5 DNA-TPC into 293 cells, the desired recombinant adenovirus was generated by homologous recombination (Figure 3). The right adenovirus clone was identified by restrictive analysis of viral genome (Figure 4), and propagated in 293 cells. The titer of the propagated 3rd recombinant adenovirus clone was 2.1×10^9pfu/ml.

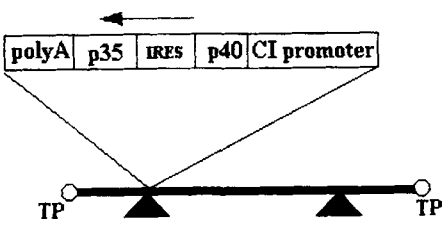

Fig. 3. Structural demonstration for the dicistronic adenovirus vector of hIL-12.

In vitro Expression of the Recombinant Adenovirus

ELISA assay showed that there was no detectable hIL-12 in the supernatants from untransfected or LacZ-transfected 293 cells or HepG2 cells or primary human skin fibroblasts. 48hr after infection with the hIL-12 recombinant adenovirus, these three kinds of cells expressed 58.5, 49.6, and 36.8ng/10^6cells/24hr of hIL-12 respectively. Furthermore, we tested the bioactivity of expressed hIL-12 with PHA-stimulated human lymphoblasts.

The results showed that the hIL-12 expressed by its recombinant adenovirus could stimulate the proliferation and IFN-γ production of human lymphoblasts (Table 1), suggesting the dicistronic adenovirus vector of hIL-12 could effectively mediate the expression of bioactive hIL-12.

Table 1. hIL-12 expressed by its recombinant adenovirus stimulated the proliferation of human PHA-stimulated lymphoblasts and their IFN-γ production

	^{3}H-TdR incorporation(cpm)	IFN-γ (pg/ml)
PBS control	2028±234	<3.0
hIL-12 standard(2ng/ml)	26745±2143*	234.8±18.6
AdLacZ-293 SN	2156±198	<3.0
AdIL12-293 SN(1:10)	33884±2976*	378.5±12.5
AdLacZ-HepG2 SN	1865±202	<3.0
AdIL12-HepG2 SN(1:10)	30984±2870*	364.8±10.6

SN: Supernatant * $p<0.01$ compared with control

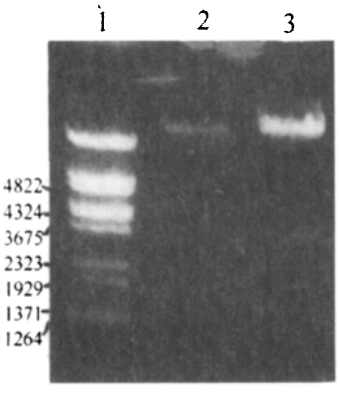

Fig. 4. Digestive analysis for the genomic DNA from the 293 cell clone producing recombinant Ad of hIL-12 .
lane 1. λ/BstE II DNA marker
lane 2. ClaI digests of hIL-12 Ad clone

DISCUSSION

Since IL-12 is a heterodimer encoded by separate genes, it's essential to express both subunits effectively in the same individual cells for producing bioactive IL-12. Up to now, there are several different approaches to achieving the co-expression of multiple genes. The first takes advantages of mRNA splicing signal to regulate splicing of a single primary transcript expressed the same promoter and generate separate mRNA. This strategy has been used to construct dicistronic retroviral vector, and it's found that the expression of one gene is usually at the expense of the other, and the splicing efficiency is various with the context. The second involves expression of different genes from different promoters, which is often used in construction of retroviral vector and in gene therapy protocols. Recently, mouse IL-12 adenovirus vector was constructed by inserting p40 and p35 cDNA into E1 and E3 region respectively.[11] In retroviral vector, this strategy sometimes is compromised by competitive interference between promoters. The third one is to use IRES, which has been used successfully to construct dicistronic and polycistronic expression vector.[8] The hIL-12 dicistronic retroviral vector has been constructed using this strategy and approved to be used in clinical trial.[12] To take advantages of adenovirus vector, we constructed dicistronic vector of hIL-12. Compared the double recombinant adenovirus vector of IL-12 constructed by inserting p40 and p35 into E1 and E3 region separately, our dicistronic adenovirus vector was constructed by inserting dicistronic expression cassette into E1 region with decreased complexity of construction and was still reserved spare room in E3 region for insertion of another gene when necessary.

Adenovirus vectors, which are capable of mediating gene transfer at higher efficiency over retroviral vector and expressing gene transiently, present an attractive alternative in gene therapy. The dicistronic adenovirus vector of hIL-12 could be transfected into fibroblasts or tumor cells *in vitro*, or delivered *in vivo* directly in cancer immunotherapy. It could also be used as an adjuvant in combination

with vaccine to treat infectious diseases. [13]

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