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World Chin J Digestol 2003 March;11(3):281-284
世界华人消化杂志 ISSN 1009-3079 CN 14-1260/R
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# 转导单链免疫毒素基因的PBMCs对人肝癌细胞SMMC-7721的体外杀伤活性

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收稿日期:2002-11-19 接受日期:2002-12-20

## *In vitro* cytotoxicity of PBMCs via genetic modification of single-chain immunotoxin

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Received:2002-11-19 Accepted:2002-12-20

## Abstract

AIM:To investigate the selective cytotoxicity of single-chain immunotoxin (sFv-TNF-α fusion proteins) in cell line SMMC-7721.

METHODS:HCC-specific killer cells were generated by transducing the recombinant retroviral virus in supernatant of the virus producing cells into human peripheral blood mononuclear cells (PBMCs). PCR and RT-PCR were used to detect integration and transcription of the sFv-TNF-α gene in transduced PBMCs (PBMCs/PST). MTT method was used to detect antitumour activity of the sFv-TNF-α fusion proteins.

RESULTS:There was integrated sFv-TNF-α gene in the genome of PBMCs/PST, and PBMCs/PST were able to express the fusion sFv-TNF-α proteins. Cell killing was significant in HCC cells co-cultivated with PBMCs/PST, whereas the PBMCs/pLXSN control cells had no significant cytotoxic effects on HCC cells.

CONCLUSION:Expression of sFv-TNF-α fusion proteins in PBMCs/PST has cytotoxicity to HCC cells *in vitro*.

Cheng H,Liu YF, Zhang HZ, Shen WA, Zhang J, Zhang J. *In vitro* cytotoxicity of PBMCs via genetic modification of single-chain immunotoxin. Shijie Huaren Xiaohua Zazhi 2003;11(3):281-284

## 摘要

目的:用携带分泌型抗肝癌单链免疫毒素基因(sFv-TNF-α)的重组逆转录病毒感染人外周血单个核细胞(peripheral blood mononuclear cells, PBMCs),使其表达并分泌针对人肝癌细胞的sFv-TNF-α融合蛋白,观察转导的PBMCs对体外培养人肝癌细胞SMMC-7721的杀伤作用.

方法:用感染性重组病毒产生细胞$C_{22}$(PA317/PST)产生的病毒上清转导人PBMCs,采用PCR和RT-PCR方法对转导的PBMCs(PBMCs/PST)进行DNA和mRNA水平的分析. PBMCs/PST与SMMC-7721共培养,MTT法检测PBMCs/PST表达产物对肝癌细胞的体外杀伤活性.

结果:PCR及RT-PCR结果显示PBMCs/PST中扩增出外源目的基因对应的电泳条带.MTT法检测结果,分泌型抗肝癌单链免疫毒素对体外培养肝癌细胞SMMC-7721的杀伤率为(38.2±6.7)%.

结论:分泌型抗肝癌单链免疫毒素基因可以在PBMCs中整合并稳定表达,其分泌的表达产物对SMMC-7721具有一定的体外杀伤作用.

程虹,刘彦仿,张惠中,沈万安,张菊,张静. 转导单链免疫毒素基因的PBMCs对人肝癌细胞SMMC-7721的体外杀伤活性. 世界华人消化杂志 2003;11(3):281-284
http://www.wjgnet.com/1009-3079/11/281.htm

## 0 引言

肝细胞肝癌(hepatocellular carcinoma, HCC)是常见的恶性肿瘤之一,位居我国恶性肿瘤发病的第三位,约占全世界肝癌病例的42.5%,每年死于肝癌的人数约为11万[1-7]. 迄今为止,肝癌的病因和发病机制仍不十分清楚,更无理想的治疗手段[8-18]. 随着现代分子生物学和免疫学的发展,自1990年代初迄今,已有百余项肿瘤基因治疗方案获准进入临床试验,但纵观近10 a的研究发现,其临床疗效与预期的目标之间还存在较大差距,原因之一就是肿瘤细胞的特异性识别尚未解决. 肿瘤特异性单链抗体的问世为基因治疗靶向性问题的解决提供了新的手段,近年来成为肿瘤免疫基因治疗中的热点,目前已有多种单链免疫毒素基因治疗计划进入临床试验[19-27]. 在本实验室成功克隆的抗肝癌单链抗体基础上,我们构建了分泌型抗肝癌单链免疫毒素基

因逆转录病毒表达载体 pLXSN-sFv-TNF-α(PST)，并用PA317细胞进行包装，筛选出一株具有较高滴度的感染性重组病毒产生细胞$C_{22}$(PA317/PST)[28]. 用$C_{22}$细胞产生的病毒上清转导人外周血单个核细胞(peripheral blood mononuclear cells，PBMCs)，使其表达并分泌针对人肝癌细胞的靶向性 sFv-TNF-α融合蛋白，观察转导的PBMCs对体外培养肝癌细胞的杀伤作用.

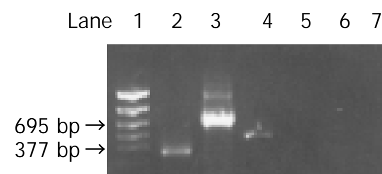
## 1 材料和方法

1.1材料 人肝癌细胞系SMMC-7721为本室保存,RPMI1640常规传代培养. 转染的逆转录病毒产生细胞系 $C_{22}$ 由本室构建[28]，用DMEM常规传代培养. DMEM，G418，Superscript™逆转录试剂盒和Trizol Reagent为Gibco公司产品，Polybrene为Sigma公司产品，MTT为Serva公司产品，PHA购自Sigma公司, 超级小牛血清购自杭州四季青生物工程材料研究所. 重组人IL-2为上海生物技术研究所产品. 淋巴细胞分离液购自上海试剂二厂.

1.2 方法 抽取正常人外周血50 mL，肝素抗凝，加入淋巴细胞分离液50 mL，室温1 400 g离心30 min，吸取单个核细胞层悬液，40 mL无血清RPMI1640洗涤细胞3次(1 000 g离心10 min)，计数,用含200 mL/L小牛血清的完全RPMI1640培养液稀释为活细胞$1\times10^9\cdot L^{-1}$,加入$5\times10^5\cdot L^{-1}$ IL-2及$5\times10^3 \mu g\cdot L^{-1}$ PHA,于37 ℃,50 mL·$L^{-1}$ $CO_2$培养3 d，换含$5\times10^5\cdot L^{-1}$ IL-2, 200 mL/L小牛血清的完全RPMI1640培养液继续培养3 d后，进行逆转录病毒的转导[29]. 用$C_{22}$细胞制备重组逆转录病毒上清[28]，取5 mL $C_{22}$细胞上清(不含G418)及8 mg·$L^{-1}$ Polybrene感染$1\times10^6$个PBMCs，置37 ℃, 50mL·$L^{-1}$ $CO_2$孵箱内培养6 h，换普通完全RPMI1 640培养液，连续感染3 d，转导的PBMCs命名为PBMCs/PST.对照组PBMCs用PA317/pLXSN(空白载体)细胞上清感染，转导的PBMCs命名为PBMCs/ pLXSN. 病毒连续感染3 d后4 d转入含500 mg·$L^{-1}$ G418的完全RPMI1 640培养液中培养1 wk. 分别提取重组逆转录病毒转导的PBMCs/PST和PBMCs/ pLXSN细胞中的基因组DNA[28]， 用3对引物(sFv-1和sFv-2, TNFα-1和TNFα-2及neo-1和neo-2), 分别扩增细胞基因组DNA中sFv, TNF-α及neo基因，以证实外源基因导入靶细胞. PCR反应于25 μL体积中完成，基因组DNA模板2.5 μL，引物2.5 μL,10×PCR缓冲液2.5 μL, 2.5mmol·$L^{-1}$，dNTP 2.5 μL，TaqDNA聚合酶0.5 μL，加水补足25 μL，混匀后在PCR仪上扩增.循环参数为:94 ℃变性60 s，58 ℃退火60 s,72 ℃延伸90 s，经30个循环后，72 ℃保温10 min.PCR产物于15 g·$L^{-1}$琼脂糖凝胶电泳,紫外透射仪观察PBMCs/PST细胞基因组DNA中的外源基因. 采用Trizol一步法, 分别提取重组逆转录病毒转导的PBMCs/PST和PBMCs/ pLXSN细胞中的总RNA. 利用Superscript™系统，以细胞内总RNA为模板进行cDNA第一链的合成:3.5 μL RNA，2 μL 5×逆转录缓冲液，1 μL Oligo($dT_{12}$),70 ℃保温10 min，冰浴2 min，加入1 μL 100mmol·$L^{-1}$ DTT，1 μL 10mmol·$L^{-1}$ dNTP，1 μL RNase inhibitor，0.5 μL MMLV逆转录酶，37 ℃反应1 h. 以cDNA第一链为模板进行sFv和TNF-α基因的扩增，PCR反应于50 μL体积中完成，cDNA模板2.5 μL，5umol·$L^{-1}$引物5 μL，10×PCR缓冲液5 μL，2.5 mmol·$L^{-1}$ dNTP 5 μL，TaqDNA聚合酶0.5 μL，加水补足50 μL，混匀后在PCR仪上扩增. PCR产物于15 g·$L^{-1}$琼脂糖凝胶电泳，紫外透射仪观察sFv和TNF-α基因的mRNA. 选择对数生长期的人肝癌细胞SMMC-7721接种于24孔培养板中，每孔$2\times10^4$，将PBMCs/PST按每孔$2\times10^5$接种于孔径0.4 μm的Millicell滤膜培养皿中， 并将Millicell置24孔培养板中，使PBMCs/PST与SMMC-7721共培养[29,30]. 设PBMCs/ pLXSN与SMMC-7721共培养为空载体对照组，PBMCs与SMMC-7721共培养为空白对照,每种细胞设20复孔. 培养板置37 ℃, 50 mL·$L^{-1}$ $CO_2$孵箱内培养，72 h后取出Millicell，加MTT每孔40 μL至24孔培养板中，继续培养4 h，弃上清，加入0.2 mL DMSO终止反应，充分振荡后在酶标仪上检测$A_{550nm}$值，计算细胞杀伤率[29].

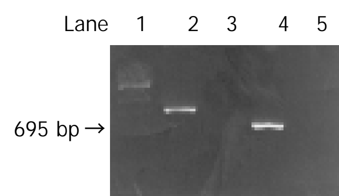
## 2 结果

2.1 PBMCs/PST基因组DNA中外源基因扩增 PCR产物电泳后观察, 可见相应的327 bp, 470 bp, 790 bp大小的条带，分别与neo, TNF-α, sFv基因片段大小相符，而PBMCs/ pLXSN中未扩增出这3种大小的条带(图1)，证实PBMCs / PST中有外源性目的基因的导入.




1 PCR markers 2 PBMCs/PST (Neo, sFv, TNF-α)
5-7 空载体对照PBMCs/pLXSN (Neo, sFv, TNF-α)
图1 基因组DNA中sFv、TNF-α及neo基因PCR扩增结果.

2.2 外源基因在PBMCs/PST中转录水平的分析 RT-PCR产物电泳后，可见相应的470 bp，790bp大小的条带，分别与TNF-α，sFv基因片段大小相符，对照组未扩增出这两种大小的条带(图2)，证实PBMCs/PST中外源性目的基因有mRNA表达.




1 PCR markers 2 PBMCs/PST (sFv) 3 空载体对照PBMCs/pLXSN( sFv)
4 PBMCs/PST(TNF-α) 5 空载体对照PBMCs/pLXSN(TNF-α)
图2 sFv和TNF-α基因mRNA的RT-PCR检测.

2.3 细胞杀伤率检测　用 MTT 法检测与 PBMCs/PST，PBMCs/pLXSN 共培养 SMMC-7721 的 $A_{550nm}$(A)值及与 PBMCs 共培养 SMMC-7721 的 $A_{550nm}$ 值($A_0$)，计算对 SMMC-7721细胞的杀伤率(%). 计算公式为:细胞杀伤率(%) = (1-A/ $A_0$)× 100 %.PBMCs/PST($n$ = 20)细胞杀伤率为(38.2 ± 6.7) %，而 PBMCs/ pLXSN($n$ = 20)细胞杀伤率为(15.7 ± 3.1) %，经 t 检验，P <0.01.

## 3 讨论

实现人类实体瘤基因治疗的途径主要有 2 种,(1)在体靶细胞的转染(in vivo)，即直接基因治疗途径，例如将细胞因子或肿瘤抗原的基因表达载体直接体内注射或将 MHC-I 基因直接注射入瘤体内等治疗肿瘤 ;(2)离体靶细胞的转染(ex vivo)，即间接基因治疗途径，必须分离并培养离体的靶细胞，并在接受基因转移后移植回人体以治疗疾病，例如应用体外基因修饰的 TIL、瘤苗、成纤维细胞等体内回输或接种的方法治疗肿瘤[31-34]. 我们选择逆转录病毒介导的体外基因转移方法进行基因治疗的研究，要求基因转移的靶细胞既要容易获得，又要容易回输到人体，因此，我们采用PBMCs作为基因转移的靶细胞. 由于目前所常用的基因转移方法[35-41] (包括逆转录病毒载体 - 包装细胞转移系统)效率均不高，获得大量的靶细胞是重要的先决条件，因此，要求该细胞易于体外培养，而且具有一定的分裂和增生能力. 此外，逆转录病毒载体对处于活跃分裂状态的细胞感染率和基因转移率较高，而对未分化的分裂不十分活跃的细胞感染率和基因转移率较低，因此，基因治疗中应尽量选择处于活跃分裂状态的细胞作为靶细胞.PBMCs 具备上述的各项条件，目前是一种较理想的基因治疗靶细胞[42-44]. 虽然有研究表明，TIL 抗肿瘤的活性是 PBMCs 的 50-100 倍，而且具有良好的肿瘤病灶趋向性和浸润性[45-49]，但由于其不易分离和培养，在某些肿瘤如肝癌中 TIL 数量极少，无法满足基因治疗中反复操作的要求，因而其应用受到很大的限制.

我们将正常人 PBMCs 分离并用 PHA 和 IL-2 进行体外刺激培养，用携带分泌型抗肝癌单链单链免疫毒素基因的重组逆转录病毒感染,使其表达并分泌针对人肝癌细胞的 sFv-TNF- α融合蛋白, 采用 PCR,RT-PCR 方法，对转导的 PBMCs 进行 DNA 和 mRNA 水平的分析，证实外源目的基因在PBMCs中整合并表达.将转导的 PBMCs 与 SMMC-7721 共培养，由于 Millicell 底部为孔径 0.4 μm 的滤膜，细胞不能通过该滤膜，而其分泌物可以相互透过[29]，因此，用 MTT 法可检测 PBMCs/PST 表达产物对肝癌细胞的杀伤作用，结果表明，分泌型抗肝癌单链单链免疫毒素对体外培养的肝癌细胞系 SMMC-7721 具有杀伤作用. 本实验结果表明，转导的人 PBMCs 可以表达并分泌特异性的抗肝癌 sFv-TNF- α融合蛋白，并且对体外培养的肝癌细胞系 SMMC-7721 具有杀伤活性. 因此，采用人 PBMCs 作为靶向细胞因子的表达细胞进行肝癌基因治疗是可行的.

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