総説

免疫学を基盤とした腫瘍制御法の開発

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要旨 細胞傷害性T細胞の誘導が可能な腫瘍関連抗原の存在が，主として *in vitro* の系において明らかになった。加えて，樹状細胞類似の高い抗原提示能を有する細胞を末梢血単球から誘導する技術も開発された。これらが相まって，種々の癌に対するワクチン療法が始まった。事実，ある種の癌細胞は生体において非自己としての特性を示し，患者のT細胞がこれらの癌細胞と反応する能力を有することもわかってきた。しかしながら，われわれは，「腫瘍細胞は非自己としての特性は有しつつも，先天免疫系が癌細胞を生体にとって危険なものと認識しないために，結果として癌細胞は免疫寛容の世界に存在している」と考えている。もし，われわれの仮説が正しいならば，癌細胞に対する特異的な免疫反応を誘導するには，先天免疫系が癌細胞を細菌やウイルスのように危険なものと認識するように先天免疫系を操作，改変する必要がある。これらの視点に基づいて，新たなワクチン療法として“免疫監視機構構築療法”を開発，実施してきた。本療法は非常に複雑であり，一般的な治療法としての社会貢献は小さいかもしれない。しかし，われわれは，基礎免疫学の理論にできるだけ忠実な治療戦略の開発こそ価値ある癌に対する免疫療法の扉を開けることができると信じている。ここでは，免疫監視機構構築療法を含む現在進行中の免疫学を基盤としたわれわれの腫瘍制御法を紹介する。

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Development of Therapeutic Strategies Based on Immunology against Tumors

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Summary

Tumor associated antigens that are able to induce cytotoxix T cells were identified mainly *in vitro* systems. In addition, recent technology has made it possible to generate dendritic cell-like cells having stronger antigen presentation ability from peripheral blood monocytes. As a result, vaccine-based therapies for various kinds of tumors have been started. In fact, some of the tumors display a “non-self” characteristic *in vivo*, and patients' T cells have the ability to react to these tumor cells. However, we speculate that tumor cells are in the world of immune tolerance because tumor cells are not judged to be dangerous by innate immunity. If our hypothesis is correct, to induce specific immunity against tumor cells, we have to modify the system so that the innate immune system recognizes that tumor cells are dangerous, similiar to bacteria or viruses. Based on these aspects, we developed a new vaccine-based therapy, i.e., immune surveillance architecture therapy. Since this therapy itself is too complicated, it may not make a very large social contribution as a general therapy. We believe a treatment strategy that obeyes the theory of basic immunology as much as possible will open a door to valuable immunotherapy against tumors. In this minireview, we introduce our ongoing studies, including immune surveillance architecture therapy, concerning therapeutic strategies based on immunology against tumors.

Key words: Tumor, Immune surveillance, Immune tolerance, Danger signal, Vaccine therapy
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はじめに

細胞傷害性T細胞（CTL）を誘導し得る腫瘍関連抗原が同定され，さらに強力な抗原提示能を有する樹状細胞様細胞が末梢血単球から誘導可能となり，種々の癌に対するワクチン療法が一斉にスタートした。実に，様々な方法が試みられているが，これらの情報に関してはPubMedを開いてほしい。ここでは，われわれ腫瘍制御学分野において研究開発型医療として開発中の免疫学を基盤とした腫瘍制御法に焦点を絞って紹介する。

I. 感染免疫の概要

免疫系は，生体にとって危険かどうかといったファジー（特異性が低い）なアナログ方式の自然免疫系と自己［0］，非自己［1］といった厳密なデジタル方式（特異性が高い）で組み立てられている獲得免疫に区別して論じられることが多かった。したがって，どちらのシステムを動かすかによって，非特異的免疫療法あるいは特異的免疫療法と呼ばれてきた。しかし，最近の分子生物学的研究成果は，自然免疫系と獲得免疫系が樹状細胞を中心とする抗原提示細胞によりつながっており，自然免疫系が危険と察知しなければ情報が正しく獲得免疫系に伝わらないことを明らかにした。つまり，厳格な獲得免疫系はファジーな自然免疫系のコントロール下にあると理解することができる。生体にとって危険かどうかは，細菌などの抗原侵入を察知したマクロファージや樹状細胞あるいはNK細胞といった自然免疫系細胞が産生するサイトカインの種類によってしばしば判断される。

たとえば，ウイルス感染の場合を想定してみよう。ウイルスが感染した細胞はIFN-αを産生する。IFN-αはNK細胞を活性化し，活性化したNK細胞はIFN-γを産生する。IFN-γはマクロファージを活性化し，活性化マクロファージはIL-12やTNF-αを産生する。IL-12はNK細胞をさらに活性化する。一方，TNF-αは感染ウイルスの情報を取り込んだ樹状細胞の成熟化を誘導する。成熟化した樹状細胞はリンパ管に侵入可能となり所属リンパ節に到達し，ウイルス感染から細胞を守るための傷害性T細胞（CTL）や抗体産生B細胞が活性化，増殖するというシナリオである。

II. 腫瘍細胞は非自己か？

まず，厳密なシステムである獲得免疫系は，癌を非自己と認識しているのか？という問題である。10年ほど前に，癌細胞に特異性の高い種々の癌関連抗原（精巣特異的抗原であるMAGE蛋白など）のペプチドが癌細胞表面のMHC class I分子（ヒトではHLA class I分子）と結合し，CD8陽性T細胞に非自己として認識されることが主として*in vitro*の系で示された。最近では，CD4陽性T細胞が認識するMHC class II分子と結合した癌関連ペプチドも次々と報告されている。これらの多数の論文は，T細胞が癌細胞を非自己として認識し得ることを示している。一方，実際の癌患者由来の腫瘍細胞に対して明らかな傷害性を発揮するT細胞を患者末梢血やリンパ節あるいは腫瘍局所から手に入れることは甚だ困難である。また，実験的にT細胞が認識可能であることが証明されている抗原ペプチドを患者の抗原提示細胞上のMHC分子に乗せて，患者のT細胞が反応するかELISPOTアッセイなどにより検討してみてもほとんど反応は確認されない。つまり，患者は大きな癌を抱えているにもかかわらず癌細胞を非自己として認識するT細胞は患者の生体には極めて少数存在しているにすぎないと考えざるを得ない。いい換えると，「T細胞は癌細胞を非自己として認識する能力をもってはいるが，臨床の場で遭遇する癌細胞は患者のT細胞によってほとんど認識されていない」可能性があるということである。これには少なくとも二つの理由が想定される。一つは，臨床癌にまで発育した癌はすべて免疫系を逃れた癌だという考えであり，二つ目は，そもそ

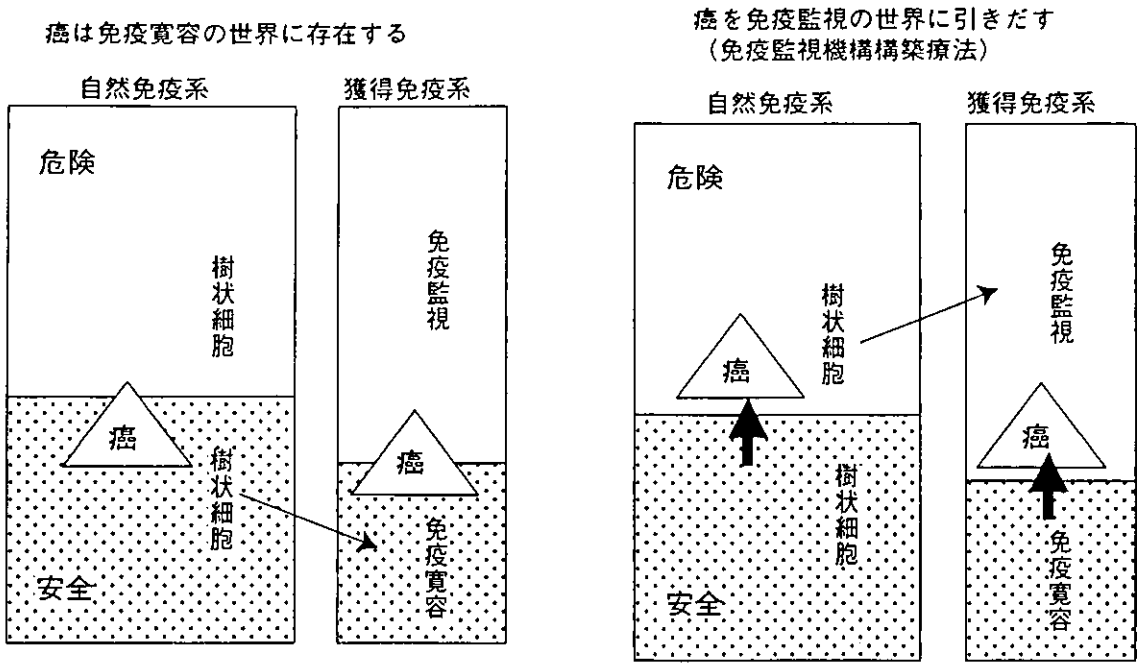

図1 腫瘍免疫の概要（仮説）
癌細胞は，獲得免疫系の世界からみれば非自己としての特性を有している。しかし，自然免疫系の世界では危険なものとは判断されない。癌が発生する組織において危険と判断されなければ，癌の情報は獲得免疫系へ伝達されない。つまり，癌に対する免疫監視機構は作動しない。もしも安全と判断されて癌の情報が獲得免疫系へ伝えられた場合は，癌に対する免疫寛容が誘導されてしまう。したがって，“免疫監視機構構築療法”の基本は癌細胞を自然免疫系に危険なものとして認識させ，さらに獲得免疫系における癌細胞の非自己性を高めることにある。そして大切なことは，生体をPSK投与や栄養改善などにより免疫系が作動しやすいように改善する努力である。

も癌は生体においては非自己というより自己として認識されているという考え方である。われわれは，後者すなわち，臨床癌は，「免疫監視機構の世界ではなく免疫寛容の世界に存在している」と考えている。

III. 腫瘍細胞は危険か？

前に述べたように，自然免疫系が危険と判断しなければ獲得免疫系すなわち免疫監視機構は動けない。では，癌局所において腫瘍細胞が危険であると自然免疫系が判断しているかという点である。特に，樹状細胞の活性化や成熟化を誘導するようなサイトカイン（IL-1，TNF-α，IFN-γ，IL-12など）が癌局所で産生されるかという問題である。なぜなら，未熟な樹状細胞は貪食による抗原捕捉力は高いが，リンパ管内への浸潤能，抗原処理能あるいはT細胞活性化に必要な補助分子の発現が弱い。われわれは，進行胃癌を対象に上記サイトカイン産生を検討したがmRNAレベルでも上記サイトカイン発現はわずか20%程度の症例で認められたにすぎなかった[1)]。一方，胃癌を含む他の癌局所では樹状細胞の成熟化を抑制するようなTGF-β，VEGFあるいはIL-10といったサイトカインの発現は約60%の症例で認められた[1-5)]。また，癌局所での癌細胞の死の形式はアポトーシスが主体と考えられるが，アポトーシス癌細胞を貪食した樹状細胞は壊死腫瘍細胞を貪食した場合に比べ，TNF-αやIL-12産生が低く成熟化の程度が弱いと予想される（Onishi, H.: 論文投稿中）。これらを総合すると，癌局所は感染局所とはまったく様相が異なり，樹状細胞が成熟化しにくい環境だと想像される。すなわち，自然免疫系は癌細胞を危険と認識していないことになる。何度も繰り返すが，自然免疫系が動かなければ獲得免疫系は動かない。

IV. 腫瘍免疫の概要（図1）

すなわち，腫瘍細胞は非自己としての特性は有

しており，T細胞は腫瘍細胞に反応する能力をもっている。しかし，腫瘍局所においては，自然免疫系が腫瘍細胞を危険なものと判断しないために腫瘍細胞の非自己としての特性（抗原性）は獲得免疫系へ伝わらない。むしろ，十分成熟化していない樹状細胞がリンパ節へ腫瘍細胞の情報を伝えている可能性があり，この場合には腫瘍細胞に対する免疫寛容（アネルギー）が誘導される。

V. 免疫監視機構構築療法

これまで，解説してきたように臨床癌は，「非自己としての特性を有してはいるが，免疫監視機構の世界ではなく免疫寛容の世界に存在している」と想像される。非自己としての特性を有するということは，癌細胞はワクチン療法の標的となり得るということになる。感染症に対するワクチン療法は非常に高い効果を上げている。したがって，腫瘍免疫の場を感染免疫の場のように変えれば効果的なワクチン療法が開発できると仮定した。われわれは，腫瘍免疫はそもそも免疫寛容の世界にあると予想しているので，再構築療法ではなく新たに感染免疫に似た免疫監視の場を作りだすという意味で構築療法と名付けた。

免疫監視機構構築療法の基本は，①生体を免疫反応誘導可能な状態に是正する。このために血清TGF-βレベルを低下させるなどの作用が期待されるPSK（クレスチン）を投与する。②腫瘍局所を炎症の場に変える。このために，少量のOK-432（ピシバニール）や活性化リンパ球を局所投与する場合がある。③自己腫瘍細胞で刺激した末梢血単球由来樹状細胞をワクチンとして使用する。原則としてOK-432あるいはOK-432刺激単核球の培養上清を用いて十分成熟化させた後，皮下投与する。④治療は2〜3週に一度，可能な限り継続する。⑤治療中にCTL前駆細胞が増加してきた時点で末梢血リンパ球を採取し，OKT-3/IL-2で活性化させ，原則として静脈内投与する。

現在，次の臨床試験が進行中である。①癌性胸腹膜炎に対する末梢血幹細胞移植および樹状細胞（DC）ワクチン療法を基本とする免疫監視機構構築療法（2001年5月，九州大学医学部倫理委員会承認）②難治性固形腫瘍に対する免疫監視機構構築療法（2002年6月，九州大学医学部倫理委員会承認）③難治性固形腫瘍に対する他家腫瘍細胞による免疫監視機構構築療法（2002年6月，九州大学医学部倫理委員会承認）などである。

この治療と並行して実施された主な基礎研究の結果を紹介すると，①癌患者の単球由来樹状細胞には細胞寿命が短く抗原提示能の低下したポピュレーションが存在する[6]。②この細胞寿命の短いポピュレーションの割合とCTL前駆細胞誘導効率は逆相関を示す（Onishi, H.: 投稿準備中）。③OK-432はGMPグレードの樹状細胞の成熟化因子であり，特にIL-12p70産生誘導作用が高い[7]。④自己腫瘍細胞を用いることで，複数の腫瘍関連ペプチドに反応するCTL前駆細胞が末梢血中に誘導される。⑤画像や腫瘍マーカーでの追跡の結果からは，抗腫瘍効果を維持するためには治療の継続の必要性が示唆される。⑥感染症や肝機能障害は本療法の効果を低下させる。

現在まで，40例を超す症例に本療法を実施してきた。phase I studyおよびphase I / II studyの結果を解析中であり[8]（Morisaki, T.: 論文投稿中）詳細は報告できないが，有害事象としては軽度の発熱，一過性の好酸球増加（1例），一過性のIgE上昇（1例）などが認められた。本療法の適応基準を満たしながらも本療法を施行しなかった症例に比較し，本療法施行例は生存期間が有為に延長しており，生存期間の延長は入院期間の延長ではなく，外来通院期間の延長の結果であることが判明している（Nakamura, M.: 投稿準備中）。

VI. 腫瘍制御学分野で開発中のプロジェクト

1. 次世代免疫療法を想定した治療用腫瘍細胞バンクの開発

腫瘍免疫療法（特に癌ワクチン療法）の分野においては，標的となる腫瘍細胞は多くの未知の腫瘍抗原を含んでおり，極めて魅力的な治療材料でもある。しかし，現存する癌細胞株の多くは，異種血清（牛胎児血清など）で培養維持されており，治療用に用いるには未知のウイルス感染の可能性および使用目的に対するインフォームド・コンセントがなされていないなどの様々な問題がある。このため，治療に特化した質の高い安全な治療用腫瘍細胞バンクを計画実施中である。現在，このバンクに登録された治療用腫瘍細胞を用いて，

「難治性固形腫瘍に対する他家腫瘍細胞による免疫監視機構構築療法（2002年6月，九州大学医学部倫理委員会承認)」を実施中である。

2. 癌細胞由来エクソゾームによるワクチン療法の開発

われわれは，単球由来樹状細胞が分泌する微細小胞（直径30～100 nm）であるエクソゾームにMHC分子やCD86などの抗原提示に必要な分子が存在しており，CD4 T細胞を活性化する能力があることを確認した（Matsumoto, K.: 論文投稿中)。さらに，癌細胞もエクソゾームを分泌し，癌細胞由来のエクソゾームには抗原ペプチドのシャペロンとして働くheat shock proteinなどの発現が確認されている。現在，これら樹状細胞由来および癌細胞由来エキソゾームを応用した新たなワクチン療法の可能性を検討中である。

3. 抗体療法と細胞療法の併用療法

HER2強陽性再発乳癌に対してHerceptin療法が保険適応され，一定の臨床効果を上げつつある。われわれは，*in vitro*の系でHerceptinがNK細胞などを介して抗体依存性細胞介在性細胞傷害作用（ADCC）を発揮することを確認した[9]。さらに興味あることには，Herceptinによる直接的な増殖抑制が認められないHER2低発現乳癌細胞に対してもADCC効果は発揮された。また，OK-432やIL-2などで活性化したリンパ球を用いた場合にも同様のADCC活性が認められる。癌組織は免疫学的に不均一な集団であり，HER2強陽性乳癌といえどもHER2発現の低い癌細胞は存在する。したがって，Herceptin療法に細胞療法を併用すれば，これらHER2発現の低い癌細胞に対する細胞傷害が発揮され，有効な治療法となることが期待される。さらに，Herceptin療法の対象をHER2過剰発現乳癌からHER2陽性乳癌に広げることも可能になるかもしれない。現在，「HER2/neu過剰発現転移性乳癌に対するHerceptin/免疫細胞療法併用療法（2003年2月，九州大学医学部倫理委員会承認)」を実施中である。

また，乳癌での臨床効果が確認されれば，HER2発現が高い膵癌あるいは胃癌へと拡大し得る可能性もある。

4. 抗癌剤（サイトカイン）および抗アポトーシス経路遮断剤併用療法

癌組織中の癌細胞は，低酸素状態，発熱，様々なサイトカイン，活性酸素など，実に多彩なストレス環境のなかで活発な増殖能を維持している。すなわち，これらストレスから自身を保護する機序（抗アポトーシス活性化経路）を有していると思われる。われわれは，「抗癌剤やサイトカインは癌細胞にアポトーシス経路の活性化と同時に抗アポトーシス経路も活性化する。したがって，抗癌剤やサイトカインの量を増やすのではなく，抗アポトーシス経路を遮断すればより低濃度でも抗腫瘍活性は増強する」という作業仮説を立て，その解析を進めてきた。われわれは，大腸癌や胃癌組織中の癌細胞において代表的な抗アポトーシス経路を形成するnuclear factor kappa B（NF-κB）が持続的に活性化されていることを見いだした[10-12]。したがって，抗癌剤やサイトカインが果たして癌細胞のNF-κBを活性化させるかを検索したところ，taxane系薬剤であるdocetaxelやサイトカインであるIFN-γが種々の癌細胞に対してアポトーシス経路と同時にNF-κB活性化を誘導することがわかった。また，これら薬剤によるNF-κB活性化を，移植医療において免疫抑制剤として臨床使用されているcyclosporin A（CsA）やFK506あるいは経口免疫賦活剤であるクレスチン（PSK）が抑制することを見いだした[13-17]。これらの基礎データを基に，CsAやFK506を投与中の移植患者の癌治療におけるIFN-γあるいはIFN-α（内因性IFN-γの誘導作用が報告されている）治療，あるいは難治性の胃癌や膵癌に対するtaxane/PSK療法およびtaxane/CsA療法の開発をめざしている。

これら薬剤によるNF-κB活性化は，単に癌のアポトーシス抵抗性に関与するだけでなく，MMP 9の活性化などを通して癌細胞の浸潤にも関与している[18]。したがって，PSKやCSAはNF-κB活性化抑制作用を通して癌の浸潤，ひいては転移を抑制することも期待され，NF-κB経路を標的とする薬剤として応用し得る可能性がある。

5. シグナルクロストークを利用した腫瘍制御

われわれは，胃癌の術前悪性度評価に基づく遺伝子カルテの作成を目標として，癌細胞の新生血管形成，浸潤や転移あるいは免疫抑制に関与する

種々の因子の胃癌組織での発現をmRNAレベルで検索してきた[4,19]。その結果，PDGF-AとTGF-βのmRNA発現程度と生存期間が逆相関を示すことや，TGF-βが浸潤能と強い相関があることなどを見いだした[20,21]。この臨床研究のデータに基づき，「TGF-βのシグナル系を直接ブロックするのではなく，癌細胞がすでにもっているTGF-βによる浸潤に関与するシグナル系とクロストークする別のシグナル系を利用することで，より生理的に浸潤を制御し得る」と仮定し，TGF-β依存性浸潤を示す胃癌の三次元培養モデルを作製し，このモデルを用いて胃癌細胞における細胞内のシグナル伝達系について解析した。その結果，TGF-βは胃癌細胞のSmad 2/3のリン酸化を引き起こし，リン酸化Smad 2/3-Smad 4複合体が核内へ移行し，MMP 9やuPAの転写が亢進し，結果として浸潤能が亢進すること，さらにはIFN-γがJak/STAT系を介してSmad 7の発現を増強させ，増加したSmad 7がTGF-βによるリン酸化Smad 2/3-Smad 4複合体の核内移行を抑制することが明らかになった[15,22]。すなわち，TGF-βによるSmadシグナル系の活性化を，IFN-γはJak/STAT系を介して抑制する可能性が示されたわけである。現在，TGF-β依存性の浸潤癌に対し，増殖抑制ではなく浸潤能抑制という「特定の悪性病態を標的としたIFN-γ療法」の可能性を解析中である。したがって，われわれはこの現象を，「シグナル系のクロストークを利用した腫瘍制御法」と名付けている。このような方法を利用することで，従来の分子標的治療とは若干異なったより生理的な治療法が開発可能だと期待している。

6. 癌局所の炎症反応制御による悪性度制御

炎症と癌の悪性度との関連性は古くから指摘されてきたが，分子レベルでの解析結果の報告は少ない。上述したように，胃癌組織においてはNF-κBが活性化しており，このNF-κB活性化と癌細胞の浸潤能には統計学的な相関が認められた[12]。一方，IL-1βをはじめとする炎症性サイトカインはマクロファージのNF-κBを活性化させる。われわれは10年以上前から，「癌とは血球様形質が再び蘇った幹細胞に近い細胞である」と考えて研究してきた。したがって，「IL-1βは癌細胞のNF-κB活性化を通して癌の浸潤能を高める」という仮説を立てて研究をスタートした。その結果，IL-1βが胃癌細胞のNF-κBを活性化し，NF-κB活性化がMMP 9の発現増強を誘導し，結果として癌細胞の浸潤能を亢進させるという仕組みが存在する可能性が明らかになった[18]。すなわち，炎症が癌細胞の悪性度を増強する可能性が分子レベルで示唆された。現在，IL-1β以外の炎症関連物質が癌細胞の種々の性質にどのような影響を及ぼすのか，果たして*in vivo*においてもこのような現象はみられるのかを動物モデルおよび臨床検体を用いて解析している。また，同時に治療法開発を目的として，癌局所での炎症の制御が果たして癌の悪性度を制御し得る可能性があるかの検討に入っている。炎症は免疫反応と強い相関があり，治療法として確立するには極めて複雑なシナリオを描く必要があるが，炎症という生体反応と癌の悪性度の関連を明らかにしていく作業は新たな治療法開発へと導いてくれると信じている。

7. 新たな視点に立った免疫化学療法

化学療法に免疫療法を組み合わせる方法は古くから行われてきた。この理由の一つは，免疫療法による上乗せ効果を当然期待したものだが，われわれは以前より，「化学療法剤のような副作用を主作用と期待して使用する薬剤の場合には，免疫療法を抗腫瘍効果の上乗せという視点とは別に，化学療法剤使用前に生体の免疫能をできるだけ整えるという視点からも実施するべきである」ことを訴えてきた。この考えに変わりはないが，これまで紹介してきたようにPSKは免疫賦活剤としての作用とは別に，NF-κB活性化抑制作用を通して癌細胞の浸潤能などに影響を与える場合がある[16,18]。さらに，種々の抗癌剤が癌細胞にNF-κB活性化を誘導し，癌の悪性化を増強させる可能性も明らかになってきた[16,17]。すなわち，PSKは癌細胞に対する浸潤能抑制剤，あるいは抗癌剤による悪性化の防止薬として作用する可能性がある。さらに，PSKが癌細胞のTGF-β産生を抑制し，癌の浸潤を抑制する可能性もある[14]。われわれが10年ほど前に報告した「大腸癌切除例に対するPSKの再発予防効果」は，この可能性を裏付けるデータかもしれない[23]。このような視点に立った新たな免疫化学療法の開発も可能であろう。

さらに，taxane系薬剤がNK細胞を中心とする免疫担当細胞の細胞傷害活性を増強することとその機序の一端が明らかとなった（Kubo, M.: 論文投稿中）。これらを総合すると，taxane系薬剤にPSKを併用した場合，PSKは癌細胞のTGF-β産生を抑制し免疫能を改善し，taxaneはNK細胞の傷害活性を高める。一方，癌細胞に対してはPSKは癌細胞のNF-κB活性化を抑制し，taxaneによる癌細胞のアポトーシス誘導を増強する。さらには，taxaneによるNF-κB活性化を通して増強された癌細胞の浸潤能をPSKは抑制するというシナリオも想定することができる。現在，難治性の固形癌を対象としたtaxane/PSK併用療法の実施作業に入っている。

おわりに

われわれは，「癌細胞を自然免疫系が危険と判断しないために，癌細胞は免疫寛容の世界に置かれている」と考えている。したがって，癌局所において自然免疫系が危険だと判断するように，癌局所を疑似感染局所に変えた上で，さらに獲得免疫誘導効率を高めるために樹状細胞ワクチン療法と活性リンパ球移入療法を併用する“免疫監視機構構築療法”といった複雑な治療法を実施している。この療法そのものは，複雑すぎて治療法としての直接的貢献は少ないかもしれない。しかし，できるだけ基礎研究のエビデンスに沿った丹念な治療を工夫することこそがより容易な治療法開発の王道であると信じている。また，免疫監視機構構築療法と並行して進行中の開発型医療研究のプロジェクトの一部を紹介した。紙面の関係上，具体的な研究成果の詳細なデータ記載は省略した。その結果，非常に観念的で雑駁な内容となったことを深く詫びなければならないが，免疫学を基盤とした新たな癌治療法を開発するための参考にしていただければ幸いである。

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ORIGINAL ARTICLE

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Monocyte-derived dendritic cells that capture dead tumor cells secrete IL-12 and TNF-α through IL-12/TNF-α/NF-κB autocrine loop

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Abstract This study focused on the question of how monocyte-derived dendritic cells (Mo-DCs) that capture dead tumor cells (Mo-DCs-Tum) secrete interleukin 12 (IL-12) and tumor necrosis factor α (TNF-α). Mo-DCs-Tum showed higher secretions of IL-12 and TNF-α than were shown by Mo-DCs. Enhanced nuclear factor-kappa B (NF-κB) activation was also induced in Mo-DCs-Tum within 6 h. The NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), suppressed both IL-12 and TNF-α secretions from Mo-DCs-Tum. Administration of recombinant TNF-α or IL-12 enhanced IL-12 or TNF-α secretion respectively in Mo-DCs-Tum. Addition of anti-TNF-α or anti-IL-12 neutralizing antibody decreased NF-κB activation and IL-12 or TNF-α secretion in Mo-DCs-Tum. These results suggest that TNF-α or IL-12 secretion induces NF-κB activation, and it stimulates further TNF-α and IL-12 secretions, i.e., an IL-12/TNF-α/NF-κB autocrine loop, in Mo-DCs-Tum. Thus, Mo-DCs-Tum secrete a large amount of IL-12 and TNF-α through accelerated NF-κB activation induced by the IL-12/TNF-α/NF-κB autocrine loop.

Keywords Dendritic cells · Human · Transcription factors · Tumor immunity

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Introduction

Dendritic cells (DCs) play a pivotal role in T and B cell–mediated immune responses [3, 5]. Recent advances in biotechnology have made it possible to generate monocyte-derived DC-like cells (Mo-DCs) in vitro from peripheral blood monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) [23]. Clinical trials of Mo-DCs pulsed with autologous tumor cells, i.e., Mo-DC–based immunotherapies, are underway for patients with a variety of malignancies including malignant melanoma, B-cell lymphoma, renal cell carcinoma, gastrointestinal carcinoma, and thyroid carcinoma [9, 13, 16, 22, 24]. However, Mo-DC–based tumor immunotherapies have had limited success. One reason is that the level of specific antigen-MHC complexes on tumors may be low and thus only a few T-cell clones that recognize tumor antigens may be activated. It has been shown that Mo-DCs which capture necrotic tumor cells (N) or apoptotic tumor cells (A) become mature, i.e., mature Mo-DCs, and they produce tumor necrosis factor α (TNF-α) and interleukin 12 (IL-12) [3]. Efficient secretion of IL-12 from Mo-DCs is helpful to overcome this problem because Th1-type helper T cells are preferentially induced by IL-12 and play an important role in the antitumor immune response [6, 8]. Unfortunately, IL-12 secretion of Mo-DCs generated from patients with advanced cancer is weaker than that of Mo-DCs from healthy volunteers [18]. These findings indicate that we should prepare Mo-DCs which secrete larger amounts of IL-12 as DC-vaccine source. However, details regarding the molecular mechanism of how Mo-DCs that capture dead tumor cells (Mo-DCs-Tum) secrete IL-12 remain unclear.

Nuclear factor-kappa B (NF-κB) is responsible for maturation of DCs and is a major regulator of the antigen-presenting function of DCs [21]. In resting cells, NF-κB is located in the cytoplasm as heterodimers of the structurally related proteins p50, p52, RelA, c-Rel, and

RelB, both of which are noncovalently associated with the cytoplasmic inhibitor, inhibitory NF-κB (IκB) [17, 25]. Activation of NF-κB is preceded by phosphorylation of IκB by IκB kinase, which is followed by proteolytic removal of IκB and movement of NF-κB to the nucleus. Nuclear translocation of NF-κB is thought to reflect activation of NF-κB [1]. After translocation to the nucleus, NF-κB stimulates transcription of cytokines such as TNF-α and IL-12 [7, 20].

In the present study, we focused on the IL-12 and TNF-α secretions from Mo-DCs-Tum and their molecular mechanism.

Materials and methods

Generation of Mo-DCs

Cultures of human peripheral blood mononuclear cells (PBMCs) were maintained in RPMI 1640 (Sanko Pure Chemicals, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Filtron Pty, Brooklyn, Victoria, Australia), 100 U/ml penicillin (Meijiseika, Tokyo, Japan), and 100 μg/ml of streptomycin (Meijiseika) (hereafter referred to as RPMI medium). Mo-DCs were generated from the adherent fraction of PBMCs as described previously [18] but with minor modifications. In brief, PBMCs were isolated from heparinized peripheral blood from healthy volunteers by Ficol-Paque (Life Technologies, Gaithersburg, MD, USA) density gradient centrifugation. PBMCs were resuspended in RPMI medium, plated at a density of 2×10^6 cells/ml, and allowed to adhere in 24-well culture plates (Nalge Nunk International, Chiba, Japan). After an overnight incubation at 37°C, the nonadherent cells were removed, and the adherent cells were harvested and cultured in RPMI medium supplemented with GM-CSF (200 ng/ml; Genetech, China) and IL-4 (500 U/ml; Osteogenetics, Wuerzburg, Germany). On day 6, nonadherent cell fractions were collected as immature Mo-DCs and examined. In the present study, Mo-DCs from ten healthy donors were prepared and used.

Induction of apoptosis and necrosis in tumor cells

A human gastric carcinoma cell line, GCTM-1, was established in our laboratory and maintained in RPMI medium [15]. Complete disruption of the necrotic cells into fragments was confirmed by light microscopy after five cycles of freezing with liquid nitrogen and thawing at 37°C. UV-triggered apoptosis of tumor cells was induced by 2 mJ/cm^2 of irradiation with a 120-mJ UVB lamp (Amersham Biosciences, Piscataway, NJ, USA). Eight hours after irradiation, induction of apoptosis was confirmed by double staining with Hoechst 33342 (Wako Pure Chemical, Osaka, Japan) and propidium iodide (PI; Sigma, St Louis, MO, USA). Under fluorescence microscopy, PI-negative and Hoechst 33432-positive cells with characteristically condensed or fragmented nuclei were defined as being apoptotic. More than 99% of UV-irradiated GCTM-1 cells had the PI-negative and Hoechst 33432-positive phenotype and typical apoptotic morphology. PI-positive cells were defined as secondary necrotic cells. Necrosis- or apoptosis-induced tumor cell cultures were centrifuged at 6,000 *g* for 10 min, the supernatant was removed, and the pellets were saved. No contamination of LPS into dead tumor cell fraction was confirmed with Limulus amebocyte lysate test (Wako, Tokyo, Japan).

Capture of dead tumor cells by Mo-DCs

Apoptotic or necrotic tumor cells were incubated with Mo-DCs at a cell ratio of 1:1 at 4°C or 37°C. Four hours after incubation, cells were collected, washed with posphate-buffered saline (PBS), and stained with Giemsa (Kokusaishinyaku, Kobe, Japan) to determine the number of Mo-DCs-Tum that captured N (Mo-DCs-Tum-N) or A (Mo-DCs-Tum-A). One hundred Mo-DCs were examined under a light microscope at ×400 magnification, and the percentage of Mo-DCs-Tum (%Mo-DCs-Tum) was calculated. Alternatively, the membrane components of N or A tumor cells were labeled with the PKH 26 red fluorescent cell linker kit (Sigma), and Mo-DCs were labeled with the PKH 67 green fluorescent cell linker kit (Sigma) according to the manufacturer's protocol. Fluorescence-labeled Mo-DCs and dead tumor cells were cocultured at an original cell ratio of 1:1 for 4 h at 4°C or 37°C, washed, and then applied to a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The fluorescence intensity was analyzed with CELLQuest software (Becton Dickinson). PKH 26-positive cells in gated Mo-DCs populations were defined as Mo-DCs-Tum-A or Mo-DCs-Tum-N.

Cytokine secretions by Mo-DCs

Ten thousand Mo-DCs and dead tumor cells obtained from 1×10^4 GCTM-1 cells were suspended in 200 μl of RPMI 1640 containing 1% human albumin. Alternatively, these cell mixtures were incubated with or without 1 μg/ml of anti-TNF-α neutralizing IgG (R&D Systems), 200 U/ml of recombinant human TNF-α (Dainippon Pharmaceutical, Osaka, Japan), 20 μg/ml of anti-IL-12 neutralizing IgG (R&D Systems), 100 μg/ml of recombinant IL-12 (Dainippon Pharmaceutical) or 100 μM of pyrrolidine dithiocarbamate (PDTC, Sigma). Twenty-four hours after the initial culture, cell-free supernatants were collected by centrifugation and stored at −80°C until use. The concentrations of IL-12 p40, IL-12 p70, and TNF-α in the culture supernatants were measured by ELISA kit specific for IL-12 p40, IL-12 p70, or TNF-α (Biosource, Camarillo, CA, USA). The detection limit of each kit was 10 pg/ml.

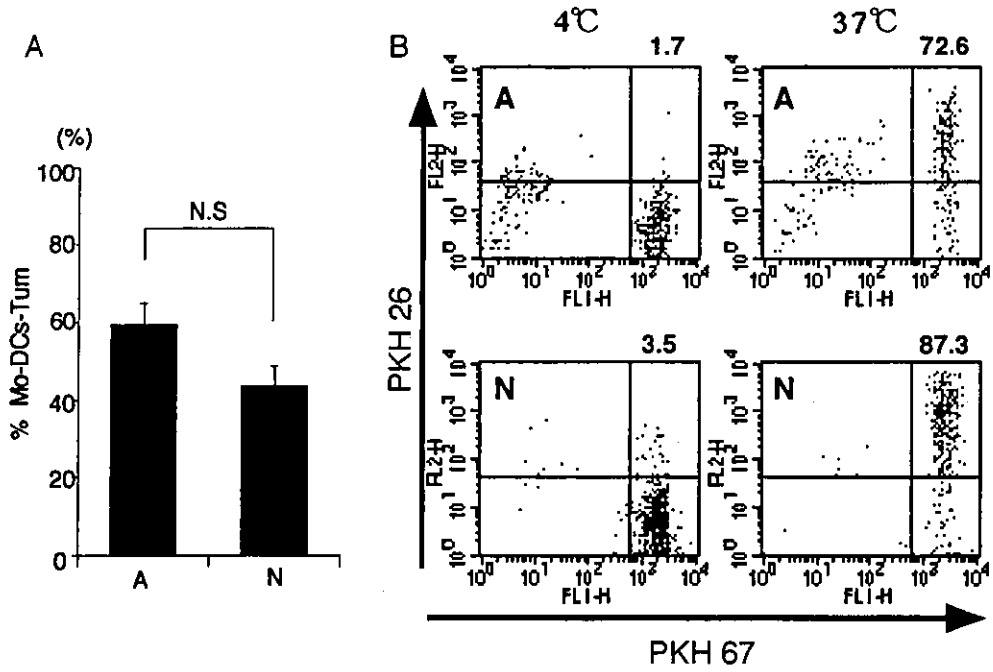


Fig. 1a,b Analysis of dead tumor cells by Mo-DCs. a Dead tumor cells were cocultured with an equivalent number of Mo-DCs for 4 h and then stained with Giemsa. One hundred Mo-DCs were examined under a light microscope, and the percentage of Mo-DCs-Tum was calculated (*%Mo-DCs-Tum*). Results are presented as mean ± SE (*bars*). The data are representative of three independent experiments using Mo-DCs generated from ten different donors. b Membrane components of dead tumor cells were labeled with PKH 26, and Mo-DCs were labeled with PKH 67. Fluorescence-labeled Mo-DCs were cocultured with dead tumor cells at a ratio of 1:1 for 4 h at 4°C or 37°C. Data are the log fluorescence intensity of Mo-DCs gated by cellular size and granulation phenotype. Fluorescence profiles of Mo-DCs-Tum at 4°C or 37°C are shown. The percentage of double-positive (PKH 26+, PKH 67+) Mo-DCs is shown in the *upper right corner* of each figure. The data are representative of three independent experiments using Mo-DCs generated from three different donors

Preparation of nuclear extract of Mo-DCs

Monocyte-derived dendritic cells cocultured with dead GCTM-1 cells for various durations with or without various agents, including anti-TNF-α neutralizing mAb, anti-IL-12 neutralizing mAb, or PDTC, were collected and washed once with PBS. Cells were then homogenized in 400 µl of hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% Nonidet P-40, and 5% protease inhibitor cocktail [0.2 mM DTT, 10 mM benzamidine, 7 µg/ml leupeptin, 50 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin, 2 µg/ml antipain, 0.7 µg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride] [Sigma]), and then incubated for 10 min on ice. Nuclei collected by centrifugation at 800 *g* for 5 min were washed once with 200 µl of hypotonic buffer and resuspended in 20 µl of low-salt buffer (20 mM HEPES [pH 7.9], 0.02 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and protease inhibitor cocktail). An equal volume of high-salt buffer (same composition as low-salt buffer but containing 800 mM KCl) was added with vortex mixing. Nuclei were incubated for 30 min at 4°C and centrifuged at 18,000 *g* for 30 min, and the supernatants were collected.

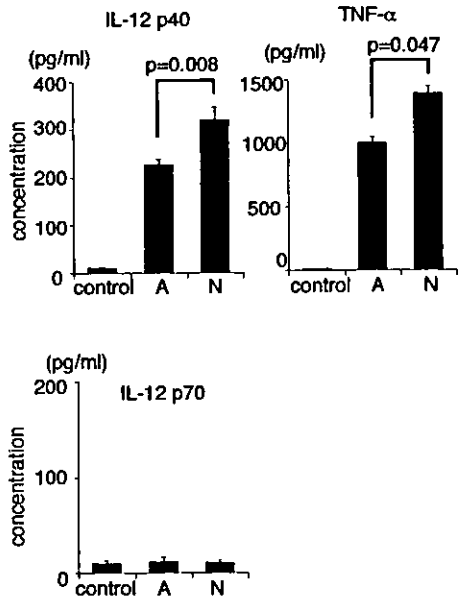


Fig. 2 Secretion of IL-12 p40, TNF-α, and IL-12 p70 by Mo-DCs. Mo-DCs (10^4) were cocultured with 1×10^5 dead GCTM-1 cells for 24 h. Cell-free culture supernatants were collected, and the concentrations of of IL-12 p40, TNF-α, and IL-12 p70 were measured by ELISA ($n=10$). Data are the mean ± SE (*bars*); *p* values for Mo-DCs-Tum-A vs Mo-DCs-Tum-N are listed. The data are representative of two independent experiments using Mo-DCs generated from ten different donors

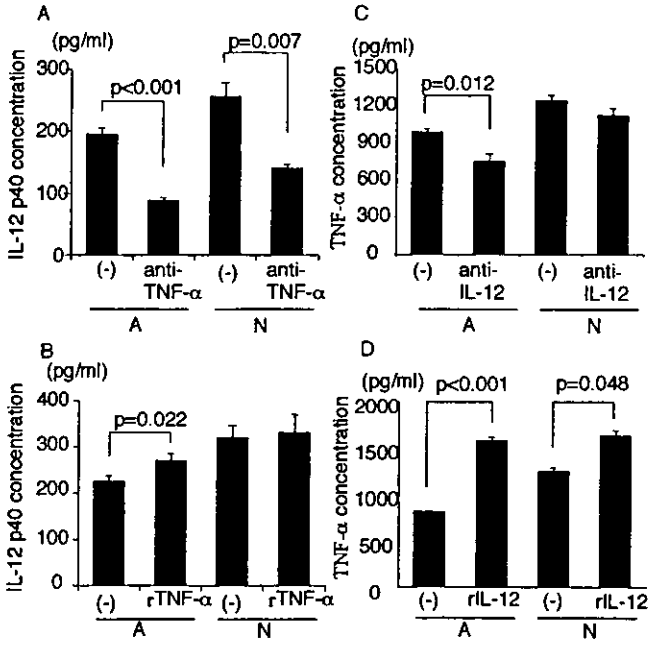


Fig. 3 **a** IL-12 p40 secretion by captured Mo-DCs in the presence of anti-TNF-α neutralizing antibody (1 μg/ml). Mo-DCs (10^4) were cocultured with 1×10^5 dead GCTM-1 cells for 24 h. Cell-free culture supernatants were collected, and the concentration of of IL-12 p40 was measured by ELISA. Data are mean ± SE (*bars*). **b** IL-12 p40 secretion by captured Mo-DCs in the presence of recombinant TNF-α (200 U/ml). Mo-DCs (10^4) were cocultured with 1×10^5 dead GCTM-1 cells for 24 h. Cell-free culture supernatants were collected, and the concentration of of IL-12 p40 was measured by ELISA. Data are mean ± SE (*bars*). **c** TNF-α secretion by Mo-DCs-Tum in the presence of anti-IL-12 (20 μg/ml). Mo-DCs (10^4) were cocultured with 1×10^5 dead GCTM-1 cells for 24 h. Cell-free culture supernatants were collected, and the concentration of TNF-α was measured by ELISA. Data are mean ± SE (*bars*). **d** TNF-α secretion by captured Mo-DCs in the presence of recombinant IL-12 (100 μg/ml). Mo-DCs (10^4) were cocultured with 1×10^5 dead GCTM-1 cells for 24 h. Cell-free culture supernatants were collected, and the concentration of TNF-α was measured by ELISA. Data are mean ± SE (*bars*). The data are representative of three independent experiments using Mo-DCs generated from ten different donors

Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts of Mo-DCs were analyzed by electrophoretic mobility shift assay (EMSA) for NF-κB nuclear translocation as described previously [11]. Briefly, nuclear protein extracts of 1×10^6 cells were incubated for 30 min at 37°C with binding buffer (60 mM HEPES [pH 7.5], 180 mM KCl, 15 mM $MgCl_2$, 0.6 mM EDTA, and 24% glycerol), poly (dI-dC) (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and ^{32} P-labeled double-stranded oligonucleotide containing the binding motif of NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, WI, USA). These mixtures were loaded onto a 4% polyacrylamide gel and separated by electrophoresis in 0.25×TBE running buffer. The oligomer-protein complexes were visualized by autoradiography.

Fig. 4 **a** NF-κB activation in Mo-DCs-Tum. Six hours after GCTM-1 cell pulsation, nuclear translocation of NF-κB in Mo-DCs was detected by EMSA. *Lane 1* Mo-DCs (control), *Lane 2* Mo-DCs-Tum-A, *Lane 3* Mo-DCs-Tum-N, *Lane 4* competition assay by the addition of 100 times NF-κB oligonucleotide. Data are the ratio of the intensity of Mo-DCs-Tum to control Mo-DCs as determined by NIH image. **b** NF-κB activation in Mo-DCs-Tum in the presence of anti-TNF-α neutralizing antibody (1 μg/ml). Twelve hours after GCTM-1 cell pulsation, nuclear translocation of NF-κB p65 in Mo-DCs-Tum was analyzed by EMSA. *Lane 1* Mo-DCs-Tum-A, *Lane 2* Mo-DCs-Tum-A in the presence of anti-TNF-α neutralizing antibody, *Lane 3* Mo-DCs-Tum-N, *Lane 4* Mo-DCs-Tum-N in the presence of anti-TNF-α neutralizing antibody. Data are the ratio of Mo-DCs-Tum with anti-TNF-α neutralizing antibody to Mo-DCs-Tum without anti-TNF-α neutralizing antibody as determined by NIH image. **c** NF-κB activation in Mo-DCs-Tum in the presence of anti-IL-12 neutralizing antibody (20 μg/ml). Twelve hours after GCTM-1 cell pulsation, nuclear translocation of NF-κB p65 in Mo-DCs-Tum was analyzed by EMSA. *Lane 1* Mo-DCs-Tum-A, *Lane 2* Mo-DCs-Tum-A in the presence of anti-IL-12 neutralizing antibody, *Lane 3* Mo-DCs-Tum-N, *Lane 4* Mo-DCs-Tum-N in the presence of anti-IL-12 neutralizing antibody. Data are the ratio of Mo-DCs-Tum with anti-IL-12 neutralizing antibody to Mo-DCs-Tum without anti-IL-12 neutralizing antibody as determined by NIH image. The data are representative of three independent experiments using Mo-DCs generated from three different donors

The intensity of the NF-κB band was estimated with the use of NIH Image version 1.60 software (NIH Division of Computer Research and Technology, Bethesda, MD, USA).

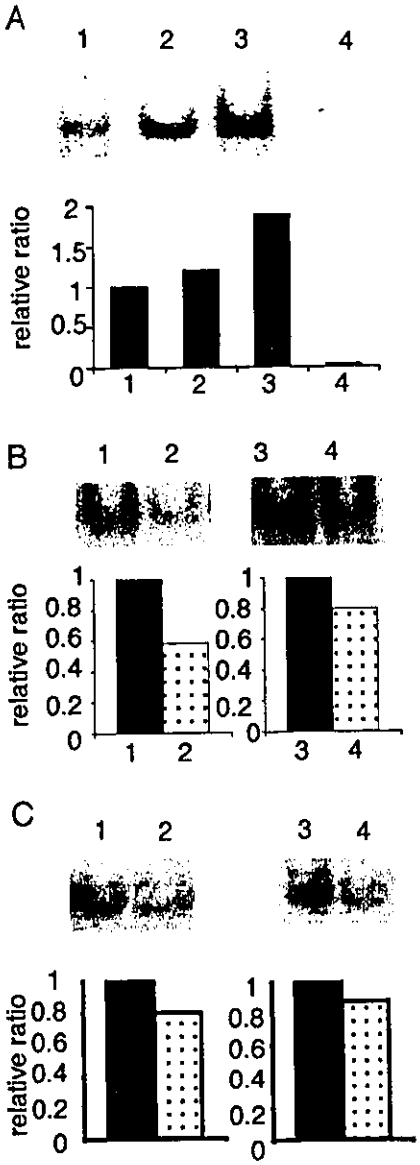
Statistical analysis

The Fisher exact probability test was used for statistical analyses. Calculations were carried out with StatView software (Abacus Concepts, Berkeley, CA, USA). All results with a p value less than 0.05 were considered statistically significant.

Results

Mo-DCs capture dead tumor cells

Capture of dead tumor cells by Mo-DCs was essentially determined with Giemsa staining. When the ratio of Mo-DCs-Tum to total Mo-DCs (%Mo-DCs-Tum) was calculated with a light microscope as described in "Materials and methods," %Mo-DCs-Tum was not affected by cellular death pattern, i.e., necrosis or apoptosis, of tumor cells (Fig. 1a). The data are representative of three independent experiments using Mo-DCs generated from ten different donors. Since evaluation with microscopy may be dependent on the investigator's subjective response, %Mo-DCs-Tum was also examined with a flow cytometer. As described in "Materials and methods," Mo-DCs and dead tumor cells were prestained with green fluorescence PKH-67



and red fluorescence PKH-26, respectively. As a result, Mo-DCs-Tum become double-positive cells (upper right rectangle in Fig. 1b). The percentage of double-positive cells in Mo-DCs-Tum-N and Mo-DCs-Tum-A was 87.3% and 72.6%, respectively. Uptake was profoundly reduced when dead cells were cocultured with Mo-DCs at 4°C, indicating that nonspecific binding of dead cells to Mo-DCs was minimal (Fig. 1b). The data are representative of three independent experiments using Mo-DCs generated from three different donors. Both Fig. 1a, b, indicate that phagocytotic ability of Mo-DCs was not affected by death pattern of tumor cells.

Mo-DCs-Tum secrete TNF-α and IL-12

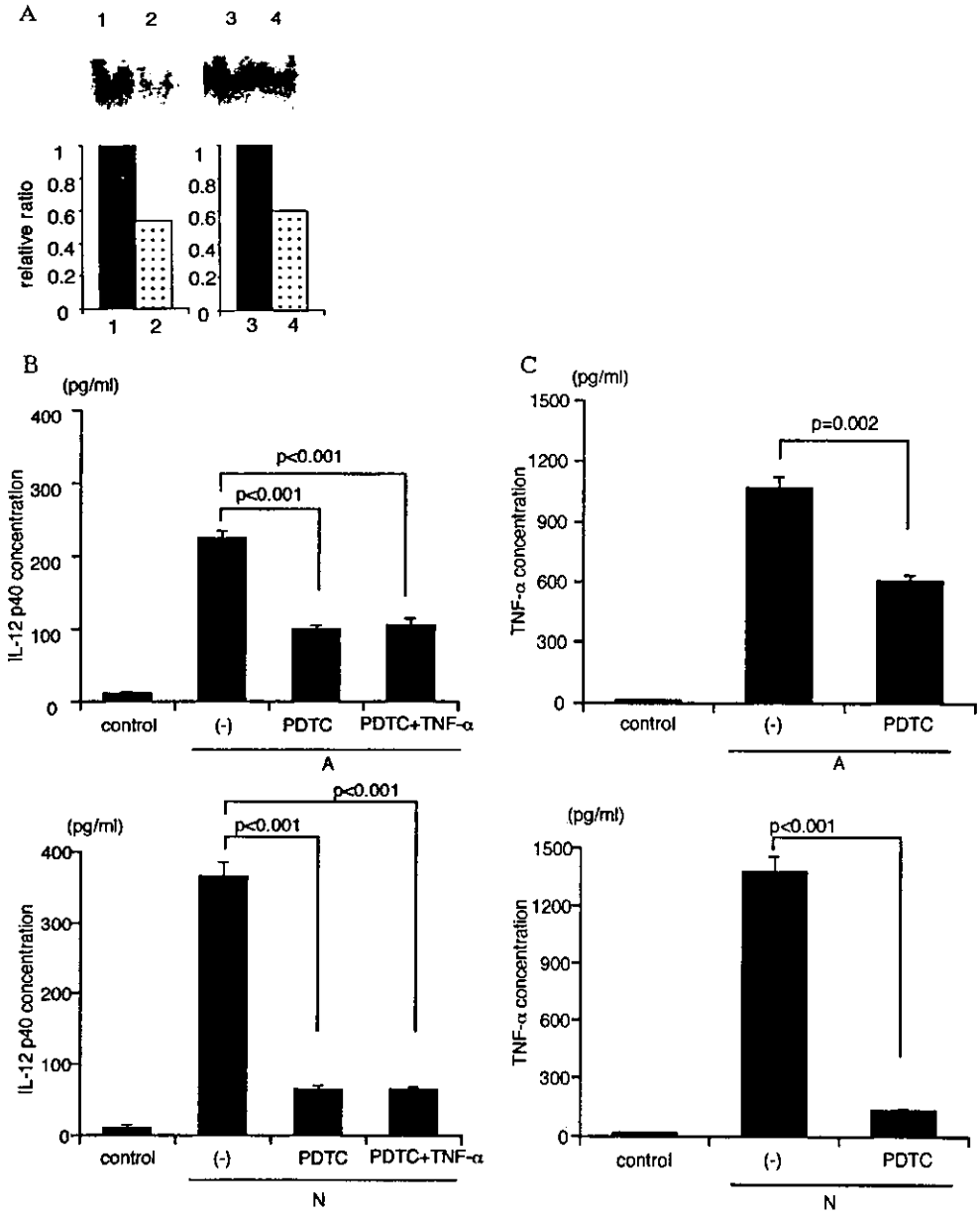
We first investigated whether Mo-DCs-Tum secrete IL-12 or TNF-α. Mo-DCs-Tum secreted both TNF-α and IL-12 p40 (Fig. 2). IL-12 p40 secretion (321 ± 26 pg/ml) by Mo-DCs-Tum-N was significantly higher than that (228 ± 10 pg/ml) of Mo-DCs-Tum-A ($p = 0.008$). TNF-α secretion (1,390 ± 60 pg/ml) of Mo-DCs-Tum-N was also significantly higher than that (1,004 ± 42 pg/ml) of Mo-DCs-Tum-A ($p = 0.047$). Concentrations of IL-12 p70 in the culture medium were below the limits of detection in both Mo-DCs-Tum-A and Mo-DCs-Tum-N. These data suggest that the death pattern of tumor cells may affect the production of these cytokines from Mo-DCs-Tum. The data are representative of two independent experiments using Mo-DCs generated from ten different donors.

TNF-α and IL-12 secreted from Mo-DCs-Tum induce further secretion of these cytokines

We next investigated the relationship between TNF-α secretion and IL-12 secretion in Mo-DCs-Tum (Fig. 3). When anti-TNF-α neutralizing antibody was added into the mixture of Mo-DCs and dead tumor cells, IL-12 p40 secretion was significantly decreased (Fig. 3a). Addition of recombinant TNF-α enhanced IL-12 p40 secretion by Mo-DCs-Tum-A but not Mo-DCs-Tum-N (Fig. 3b). Similarly, when anti-IL-12 neutralizing antibody was added into the mixture of Mo-DCs and A, TNF-α secretion was significantly decreased. However, TNF-α secretion by Mo-DCs-Tum-N has not altered significantly (Fig. 3c). Addition of recombinant IL-12 significantly enhanced TNF-α secretion by Mo-DCs-Tum (Fig. 3d). Neither cell viability nor total cell number was changed significantly by the assay (data not shown). These data suggest that TNF-α and IL-12 stimulate each other's cytokine production, i.e., TNF-α/IL-12 autocrine loop. The data are representative of three independent experiments using Mo-DCs generated from ten different donors.

Both IL-12 and TNF-α secreted from Mo-DCs-Tum induce NF-κB activation in Mo-DCs

Since both IL-12 and TNF-α are target genes of NF-κB, induction of NF-κB activation in Mo-DCs-Tum was examined (Fig. 4). NF-κB activation was estimated as translocation of NF-κB p65 to the nuclei of Mo-DCs by EMSA. Enhanced nuclear translocation of NF-κB was observed in Mo-DCs-Tum. Nuclear translocation of NF-κB was stronger in Mo-DCs-Tum-N than Mo-DCs-Tum-A (Fig. 4a). In the presence of anti-TNF-α neutralizing antibody, nuclear translocation of NF-κB in Mo-DCs-Tum was suppressed (Fig. 4b). In the presence of anti-IL-12 neutralizing antibody, nuclear translocation of NF-κB in Mo-DCs-Tum was also suppressed (Fig. 4c). These data suggest the close relationship between TNF-α, IL-12, and NF-κB. The data are representative of three independent experiments using Mo-DCs generated from three different donors.



NF-κB inhibitor PDTC suppresses both TNF-α and IL-12 secretion by Mo-DCs-Tum

To confirm the relationship between TNF-α, IL-12, and NF-κB activation, a specific NF-κB inhibitor PDTC was used. In the presence of PDTC, nuclear translocation of NF-κB in Mo-DCs-Tum was certainly suppressed (Fig. 5a). The data are representative of three independent experiments using Mo-DCs generated from three different donors. IL-12 p40 secretion by Mo-DCs-Tum in the presence of PDTC was decreased significantly in comparison to that of Mo-DCs cultured in the absence of PDTC (Fig. 5b; $p < 0.001$). The data are representative of three independent experiments using Mo-DCs generated from ten different donors. Addition of recombinant TNF-α could not ameliorate the decreased IL-12 secretion in PDTC-treated Mo-DCs-Tum (Fig. 5b). Similarly, TNF-α secretion by Mo-DCs-Tum-N or Mo-DCs-Tum-A in the presence of PDTC was decreased significantly in comparison with that in the absence of PDTC ($p < 0.001$ and $p = 0.002$, respectively; Fig. 5c). The data are representative of three independent experiments using Mo-DCs generated from ten different donors. These data indicate the TNF-α/IL-12 / NF-κB autocrine loop in Mo-DCs-Tum.

Discussion

We showed that Mo-DCs-Tum secrete considerable amounts of IL-12 and TNF-α through the IL-12/TNF-α/NF-κB autocrine loop.

◀

Fig. 5 a NF-κB activation in Mo-DCs-Tum in the presence of 100 μM of PDTC. Six hours after the GCTM-1 cell pulsation, nuclear translocation of NF-κB in Mo-DCs was examined by EMSA. *Lane 1* Mo-DCs-Tum-A, *Lane 2* Mo-DCs-Tum-A treated with 100 μM PDTC, *Lane 3* Mo-DCs-Tum-N, *Lane 4* Mo-DCs-Tum-N treated with 100 μM PDTC. Data are the ratio of Mo-DCs-Tum in the presence of PDTC to Mo-DCs-Tum in the absence of PDTC as calculated by NIH image. The data are representative of three independent experiments using Mo-DCs generated from three different donors. b IL-12 p40 secretion by Mo-DCs-Tum treated with 100 μM of PDTC with or without 200 U/ml of recombinant TNF-α. Mo-DCs (10^4) were cocultured with 1×10^5 dead GCTM-1 cells for 24 h. Cell-free culture supernatants were collected, and the concentration of TNF-α was measured by ELISA. Data are mean ± SE (*bars*). The data are representative of three independent experiments using Mo-DCs generated from ten different donors. c TNF-α secretion by Mo-DCs-Tum in the presence of 100 μM of PDTC. Mo-DCs (10^4) were cocultured with 1×10^5 dead GCTM-1 cells for 24 h. Cell-free culture supernatants were collected, and the concentration of TNF-α was measured by ELISA. Data are mean ± SE (*bars*). The data are representative of three independent experiments using Mo-DCs generated from ten different donors

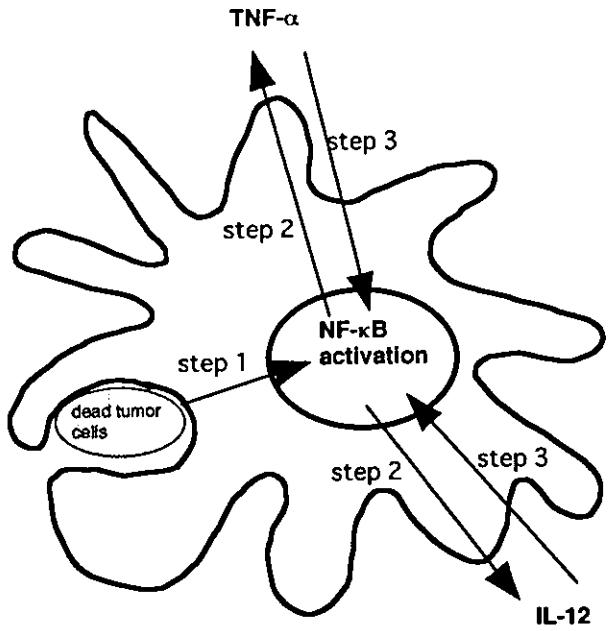


Fig. 6 Possible model of IL-12 and TNF-α secretions through IL-12/TNF-α/NF-κB autocrine loop. NF-κB activation is induced in Mo-DCs-Tum (*step 1*). NF-κB activation induces secretion of both TNF-α and IL-12 (*step 2*). Both IL-12 and TNF-α secretions induce further NF-κB activation (*step 3*). Enhanced NF-κB activation induces further secretion of IL-12 and TNF-α. Thus, IL-12, TNF-α, and NF-κB form an autocrine loop in Mo-DCs-Tum

We first showed a possibility that TNF-α secreted from Mo-DCs-Tum induces further IL-12 secretion from them, and similarly IL-12 secreted from captured Mo-DCs induces further TNF-α secretion from them (Fig. 3). These findings lead to speculation regarding the existence of a TNF-α/IL-12 autocrine loop in Mo-DCs-Tum. Antibodies against TNF-α or IL-12, however, could not completely suppress the secretion of these cytokines. In addition, recombinant TNF-α did not induce significant IL-12 secretion from Mo-DCs-Tum-N. Therefore, our data also indicate that some other mechanisms different from the TNF-α/IL-12 autocrine loop play roles in cytokine secretion from Mo-DCs-Tum. Why do Mo-DCs-Tum secrete both TNF-α and IL-12? It has been shown that transcription of TNF-α is regulated by NF-κB activation [20] and that TNF-α acts in an autocrine manner to induce NF-κB activation in Mo-DCs [23]. In addition, it has been reported that IL-12 secretion of DCs from mice with a double knockout of the NF-κB subunits p50 and cRel was impaired [19]. IL-12 is also a target gene of NF-κB in humans [7]. These data indicate that NF-κB activation plays a key role in secretion of both TNF-α and IL-12 in Mo-DCs-Tum. This possibility may be strongly supported by data showing that an NF-κB inhibitor, PDTC, significantly reduced secretion of both TNF-α and IL-12 in Mo-DCs-Tum (Fig. 5). The next question is what the initial NF-κB activation factor is. Many factors such as lipopolysaccharides, microbial stimuli, viral infection, proinflammatory cytokines, double-stranded DNA, and heat shock proteins (HSPs) have been reported to be activators of NF-κB [2, 4, 10, 14, 26–28]. We have shown that both secretion of IL-12 and activation of NF-κB induced by a streptococcal preparation OK-432 were suppressed when Mo-DCs were pretreated with an inhibitor of endocytosis, cytochalasin B [12], suggesting a possibility that NF-κB activation may be induced in Mo-DCs by capture of dead tumor cells. We are now speculating on at least two possibilities. One possibility is that capture of dead tumor cells induces first TNF-α secretion and then TNF-α secretion stimulates NF-κB activation. Another possibility is that the capture of dead tumor cells directly induces NF-κB activation.

In conclusion, it seems likely that captured Mo-DCs secrete a large amount of IL-12 and TNF-α through the IL-12/TNF-α/NF-κB autocrine loop (Fig. 6). Briefly, NF-κB activation is induced in Mo-DCs-Tum (step 1). NF-κB activation induces secretions of both TNF-α and IL-12 (step 2). Both IL-12 and TNF-α secretions induce further NF-κB activation (step 3). Enhanced NF-κB activation induces further secretion of IL-12 and TNF-α. Thus, IL-12, TNF-α, and NF-κB form an autocrine loop in Mo-DCs-Tum. Such an enhanced NF-κB activation in Mo-DCs-Tum induces them to secrete a large amount of IL-12 and TNF-α. However, it is still unknown to what degree the IL-12/TNF-α/NF-κB autocrine loop plays a role in IL-12 and TNF-α secretions from Mo-DCs-Tum. Identification of an initial signal or key molecule (step 1 in Fig. 6) operating the IL-12/TNF-α/NF-κB autocrine loop might provide new insights that could foster development of an effective antitumor immunotherapy.

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Case report

Dendritic-cell therapy after non-myeloablative stem-cell transplantation for renal-cell carcinoma

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Treatment of advanced renal-cell carcinoma is generally by nephrectomy before systemic therapy, even in patients with metastatic disease. Most patients with metastasis are treated with interferon alfa and interleukin 2 after surgery because advanced renal-cell carcinoma is highly resistant to chemotherapy and radiotherapy. However, studies have shown that non-myeloablative allogeneic peripheral-blood stem-cell transplantation leads to a graft-versus-tumour effect in patients with metastatic renal-cell carcinoma.[1,2]

A 56-year-old man underwent a radical nephrectomy for a right renal tumour in July, 1998. Histopathological analysis showed renal-cell carcinoma of clear-cell type. 2 years after surgery, the patient developed metastasis in the lung and bone, and was subsequently given immunotherapy with interferon alfa and interleukin 2. However, the metastasis progressed during immunotherapy and the patient underwent a non-myeloablative allogeneic peripheral-blood stem-cell transplantation in October, 2002. The day of infusion of donor cells was called day 0 in this protocol. The

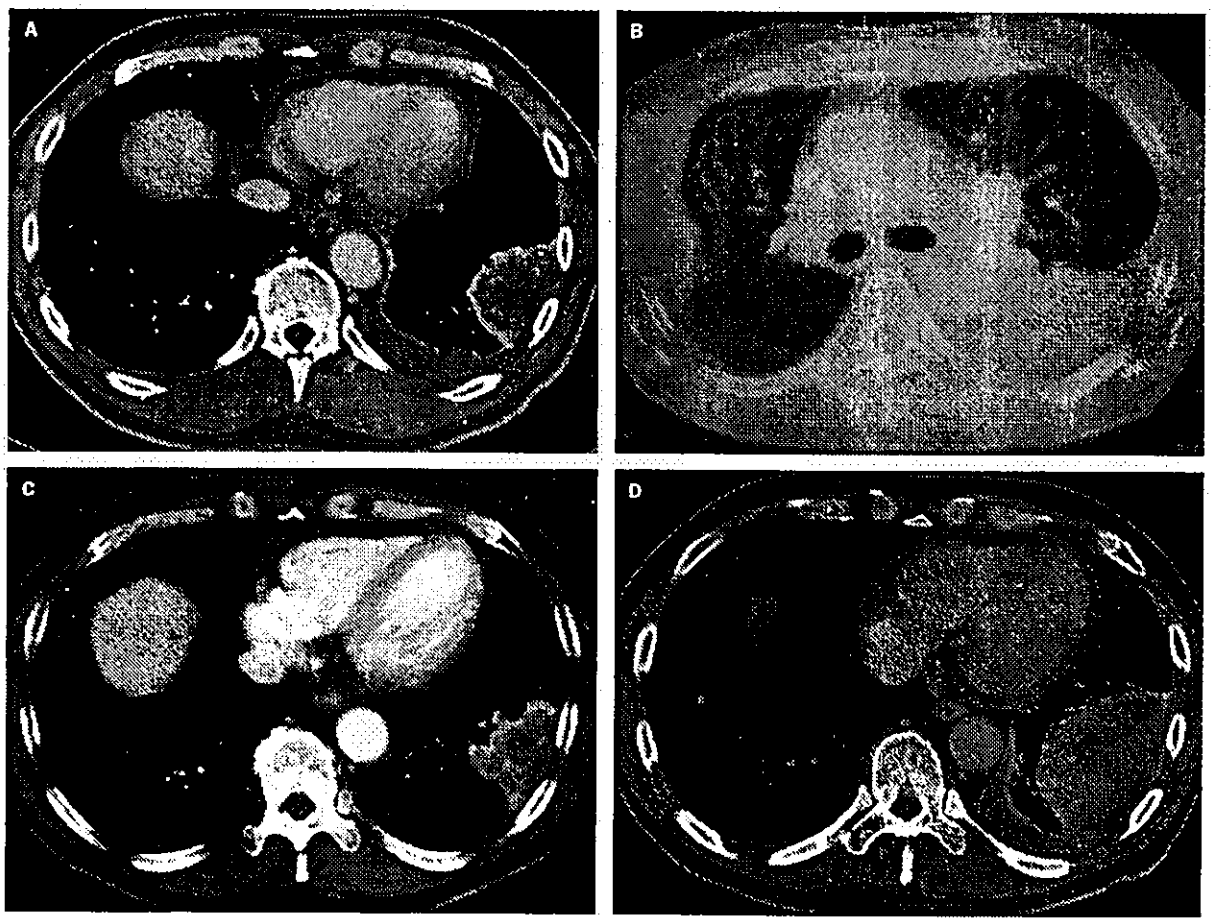

Figure 1. Clinical course of transplantation. A: Lung metastasis and pleural effusion before transplantation. B: Chest CT scan shows ground glass opacity 72 days after transplantation. C: No presence of pleural effusion or lesion 155 days after transplantation. D: Tumour growth 340 days after transplantation.

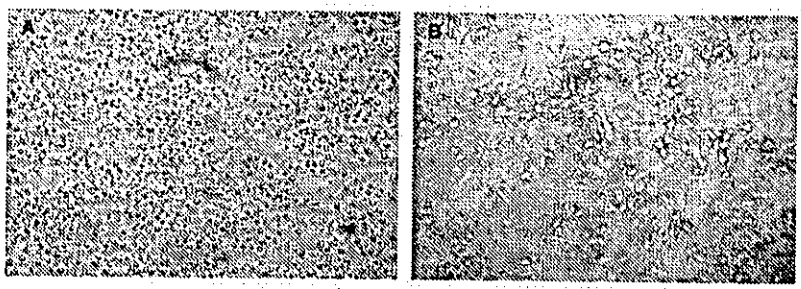

Figure 2. Microscopic analysis of monocytes after stimulation with tumour necrosis factor alfa and dinoprostone. Stimulated monocytes from the patient (A) show no dendritic shape compared with donor monocytes (B).

patient received 30 mg/m² per day fludarabine from day 8 before donor-cell infusion to day 3 before infusion; 4 mg/kg busulfan on days 6 and 5 before infusion; 3 mg/kg ciclosporin the day before infusion; and an infusion of 5×10^6/kg CD34-positive cells on the day of infusion from a donor sibling with a mismatch at a single HLA locus. 5 mg/m² methotrexate was given on days 1, 3, and 6 after donor-cell infusion and the patient also received 3 mg/kg ciclosporin daily after transplantation to prevent acute graft-versus-host disease.

The patient showed mixed chimerism for a short period and as a result we lowered the concentration of ciclosporin, which resulted in the onset of graft-versus-host disease. 70 days after the transplantation, the patient had dry eyes, a cough, and dyspnoea. Chest CT showed ground glass opacity and pleural effusion compatible with interstitial pneumonia (figure 1B), which was treated by steroid-pulse therapy (1 g methylprednisolone for 3 days). After the onset of severe graft-versus-host disease, the recipient's peripheral-blood cells changed completely into donor-type cells. Although the tumour continued to grow for 3 months after myelosuppressive treatment, tumour effusion and pleural effusion decreased after the onset of graft-versus-host disease (figure 1A and 1C). However, to control the graft-versus-host disease, the patient continued immunosuppressive therapy with 5–15 mg predonisolone daily and 25 mg ciclosporin daily for over 1 year, the tumour regrew 11 months after transplantation (figure 1D).

Interferon alfa or interleukin 2 has been given to patients who had progressive disease after transplantation[1] In some patients who receive immunosuppressive treatment to control severe graft-versus-host disease, use of interferon alfa and interleukin 2 after transplantation might enhance the graft-versus-host disease elicited by the allograft. Several clinical trials[3,4] have established the safety and feasibility of immunotherapy with dendritic cells, which can elicit specific antitumour immune responses and some clinically meaningful responses. Furthermore, no serious adverse effects or clinical signs of an autoimmune reaction were recorded in these trials. Therefore, we attempted give this patient immunotherapy by use of dendritic cells.

This immunotherapeutic technique used monocytes extracted from peripheral-blood cells of the patient. At the time of monocyte extraction, laboratory analysis showed a high concentration of C-reactive protein (72·4 mg/L) and mild anaemia (red-cell count $3\cdot0\times10^{12}$/L, haemoglobin 1·37 mmol/L). White-cell count was within the normal range ($7\cdot07\times10^9$/L). All monocytes from the patient were thought to be derived from the donor cells because complete chimerism had been already established in the patient. Monocytes were cultured in RPMI1640 medium supplemented with 5% human serum albumin in the presence of 100 µg/L molgramostim and 50 µg/L interleukin 4 for 7 days. The culture medium was changed on day 3. On day 7 the dendritic cells were pulsed with 100 mg/L autologous tumour lysate overnight and stimulated with 200 U/mL tumour necrosis factor alfa and 1 mg/L dinoprostone for 2 days. Because preliminary experiments showed that the maturation of dendritic cells from the monocytes of the patient was inadequate, we compared the dendritic-cell maturation of the patient's cells with that of the donor cells. Analysis of the shape and phenotypes of the cultured cells after stimulation with tumour necrosis factor alfa and dinoprostone showed that stimulated monocytes from the patient were smaller than those from the donor, and that no dendrites were present after stimulation (figure 2A). We then confirmed the phenotype of the cells by flow cytometry: staining of stimulated cells with CD83 molecules (monocyte markers), and analysis of forward scatter and side scatter showed a lower number of large cells such as dendritic cells in the patient's sample than in the donor sample for CD83-positive cells. Mature dendritic cells usually highly express MHC class II molecules and costimulatory molecules such as CD80 or CD86. However, the CD83-positive cells from the patient showed low expression of MHC class II, CD80, and CD86 after stimulation (figure 2B and figure 3). These results suggest that monocytes from the patient poorly differentiated into dendritic cells. Thus, we scheduled immunotherapy with dendritic cells derived from the donor's peripheral-blood cells. $1\cdot0–3\cdot9\times10^7$ mature dendritic cells pulsed with tumour lysate (100 mg/L) were given intradermally every week for 5 weeks and every other week for a further 4 weeks. The patient had no serious adverse effects apart from high-grade fever of 39°C after injection. Although immunotherapy with dendritic cells was safe for this patient, we could not inhibit the tumour growth without inducing

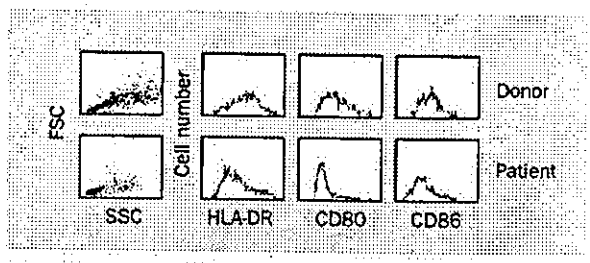

Figure 3. Phenotype of CD83-positive cells after stimulation. Number of large cells with a dendritic-cell phenotype from the patient was lower than that from the donor according to forward scatter (FSC) and side scatter (SSC). CD83-positive cells from the donor highly expressed HLA-DR, CD80, and CD86 after stimulation.

delayed-type hypersensitivity by the injection of dendritic cells pulsed with tumour lysate. The patient died of lung metastasis 3 months after initiation of dendritic-cell therapy.

There seems to be some difficulties regarding immunotherapy for patients with graft-versus-host disease, even with use of donor dendritic cells. A previous study[5] showed that steroid treatment inhibits dendritic-cell function in vitro. In our patient, immunosuppressive treatments could have suppressed antigen presentation or T-cell stimulation by dendritic cells; T-cell function also might have been suppressed. In conclusion, effective immunotherapy with dendritic cells for patients with graft-versus-host disease who have undergone allogeneic peripheral-blood stem-cell transplantation can be problematic.

Conflict of interest

We declare no conflicts of interest.

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Dendritic cell-based immunotherapy of cancer with carcinoembryonic antigen-derived, HLA-A24-restricted CTL epitope: Clinical outcomes of 18 patients with metastatic gastrointestinal or lung adenocarcinomas

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Abstract. We conducted a clinical study of cancer vaccine therapy with dendritic cells (DCs) and HLA-A24-restricted carcinoembryonic antigen (CEA)-derived peptide to assess the feasibility and efficacy of such therapy. Eighteen patients with CEA-expressing metastatic gastrointestinal or lung adenocarcinomas who were positive for human leukocyte antigen (HLA)-A24 were enrolled. DCs were generated from the patients' autologous monocyte-enriched fractions of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells in the presence of granulocyte/macrophage colony-stimulating factor and interleukin-4. The generated DCs were pulsed with CEA-derived, HLA-A24-restricted 9-mer peptide (CEA652) and injected into the patients intradermally and subcutaneously every 2 weeks. Toxicity and clinical and immunological responses were closely monitored in each patient. No severe toxicity directly attributable to the treatment was observed, and the vaccine was well tolerated. Although no definite tumor shrinkage occurred in any patient, long-term stable disease or marked decreases in the serum CEA level were observed in some patients after therapy. Most of the patients in whom treatment was clinically effective showed a positive skin response to CEA652-pulsed DCs (delayed-type hypersensitivity skin test) and a positive *in vitro* CTL response to CEA652 peptide after therapy. We conclude that active specific immunotherapy using DCs pulsed with CEA652 is a safe and feasible treatment that is clinically effective in some patients with metastatic gastrointestinal or lung adenocarcinomas. Our results will hopefully encourage further refinement and development of DC-based immunotherapy with HLA-A24-restricted CEA-derived peptide for refractory solid cancers that express CEA.

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Key words: cancer vaccine therapy, carcinoembryonic antigen (CEA), dendritic cell, gastrointestinal cancer, lung cancer

Introduction

Gastrointestinal and lung adenocarcinomas are common malignancies and a major cause of cancer-related mortality. Many patients who undergo surgery for these diseases remain at high risk for recurrence. However, there is no definitive therapy, including chemotherapy and radiotherapy, for advanced or recurrent gastrointestinal and lung adenocarcinomas. Thus, novel therapeutic strategies for these diseases are being actively pursued.

Carcinoembryonic antigen (CEA) is a 180-kDa oncofetal glycoprotein that is overexpressed in most gastrointestinal and lung adenocarcinomas and widely used as a serological marker of these cancers. It is also an adhesion molecule that plays a role in metastasis by mediating the attachment of tumor cells (1). CEA is thus considered a suitable target molecule for the development of specific immunotherapy. A cytotoxic T lymphocyte (CTL) response against CEA has been demonstrated in clinical studies of vaccinia-CEA vaccine, and a human leukocyte antigen (HLA)-A2.1-restricted CTL epitope peptide of CEA, CAP-1, has been identified (2). In 1998, a CEA-derived 9-mer peptide, CEA652 (TYACFVSNL), was identified as a potent CTL epitope restricted with HLA-A24, which is the most common HLA class I allele in the Japanese population (present in 60-65%) (3).