表1　癌の免疫学的逃避機構

1. Alteration of MHC class I and tumor antigen expression
2. Dysregulated expression of adhesion/accessory molecules by tumor and/or antigen-presenting cells
3. Secretion of immunosuppressive soluble factors either by tumor cells or infiltrating T cells or both
4. Induction of immune unresponsiveness via anergy induction or clonal deletion of responding T cells
5. Induction of suppressor cells
6. Changes in T-cell signal transduction molecules
7. Tumor utilization of products of stimulated leukocytes, ie immunostimulation of cancer

（文献[44]より改変後引用）

また，癌の免疫細胞療法の開発研究を進めていく上において，本邦と欧米との背景の違いとして最も認識すべきことは，人種差による罹患癌腫の構成比率の違いである。白人において皮膚癌は最も罹患頻度の高い癌腫であり，メラノーマはその中核をなす。しかし本邦においてはその発生頻度は低い（人口10万人当たり年間約1.5人の発生）。ヒト癌腫のなかで最も免疫原性が高く，免疫療法に反応しやすいメラノーマが罹患患者数の多い欧米で，新たなる免疫細胞療法開発のモデルかつ標的疾患として莫大な研究費が投入されることは妥当であるが，本邦においては疑問である。消化器癌や肺癌にメラノーマに対するのと同じ免疫療法を施行しても，その多くは報われないことを歴史は如実に示してきた。したがって，本邦においてはメラノーマにとらわれない免疫療法技術の開発姿勢も大事である。

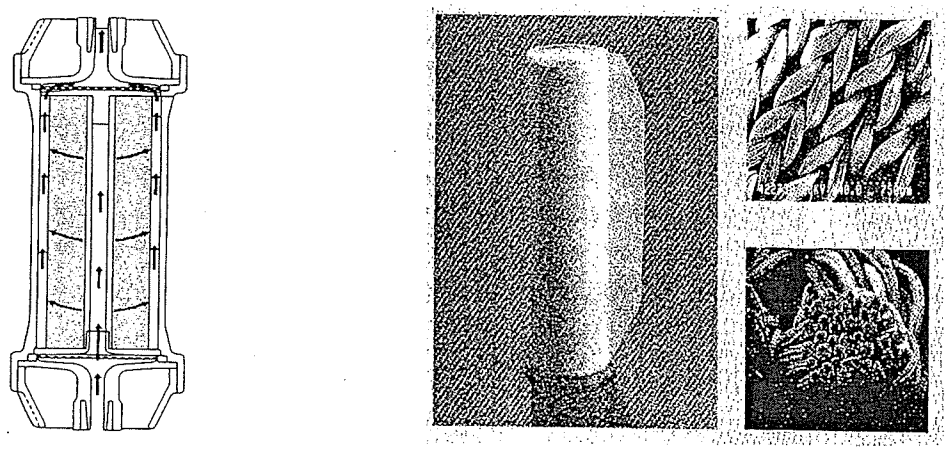
以上述べたような本邦における癌の免疫細胞療法に関する諸問題を鑑み，現在われわれが取り組んでいる*ex vivo*での細胞処理を必要としない完全閉鎖系体外循環治療による免疫細胞療法の開発研究について概説する。

免疫細胞療法，特に能動免疫療法から癌が逃れ治療が奏効しない機序は“免疫学的逃避機構”と称され，様々な要因から成り立っている[42]。表1にその代表的なものを列挙したが，腫瘍の増殖・進展に伴い担癌患者の血中に著しく増加してくる癌細胞由来あるいは宿主免疫担当細胞由来の種々の免疫抑制性の液性因子は，その制御が困難で細胞性免疫能の低下を引き起こし，その結果腫瘍の増殖はいっそう助長される。われわれが化学療法不応性の進行消化器癌，肺癌を対象として行ったDCとCEA由来ペプチドを用いたワクチン療法の臨床試験においても，治療後に血清CEA値が一度も低下せず病状の悪化を来した無効症例10例においては，血清CEA値の低下を来した有効症例8例に比し，治療開始時の末梢血リンパ球PHA幼若化能が有意に減弱しており，血清免疫抑制性酸性蛋白（immunosuppressive acid protein, IAP）が高い傾向を認めた（表2）。すなわち，能動免疫療法であるDCワクチンが有効に作動するには，宿主の基礎免疫能が温存されていることが必須条件であり，DCの成熟化などの改良を行ってもその克服が難しいことを明らかにした[43-45]。この結果を背景に2002年から進行癌患者の血中に増加する免疫抑制物質の除去を目的とした体外循環治療用カラムの開発研究を開始した。すなわち，免疫抑制物質吸着性極細繊維カラムを用いた，血漿交換を伴わない，安全で，簡便で，繰り返し施行可能な癌体外循環治療技術の開発研究である（図1）。

免疫抑制性の液性因子として，IAPやトランスフォーミング増殖因子β（transforming growth factor-β, TGF-β），IL-6，血管内皮細胞増殖因子（vascular endothelial growth factor, VEGF）などがあるが，とりわけTGF-βは免疫抑制の元凶となる中心的存在である[46]。われわれはポリスチレン系極細繊維に種々のアミノ基を付加してスクリーニングすることにより，TGF-β吸着剤を同定し，同吸着剤がVEGF，IL-6の吸

表 2 CEA-DC ペプチドワクチン療法の臨床試験における血清 CEA 値の治療後推移からみた有効症例と無効症例の治療開始時の免疫能の比較

		有効症例（n=8）		無効症例（n=10）
末梢血リンパ球数（/μl）		1,372	NS	1,162
末梢血リンパ球/好中球比		0.46	NS	0.44
末梢血リンパ球サブセット				
CD3（%）		63.5		68.5
CD16（%）		20.9	NS	21.7
CD4/CD8 比		2.21		2.05
末梢血リンパ球 PHA 幼若化能	(cpm)	86,320±27,637		71,985±25,265
	(SI)	148.5±73.4	p<0.01	71.4±27.3
血清 IAP 値（μg/ml）		584.4±279.2	NS	726.5±305.5



進行固形腫瘍患者を対象として
①免疫細胞療法との連動による効果増強
②化学療法との併用による効果増強 → 難治性癌（膵癌，スキルス胃癌など）を対象とした癌治療用医療機材としての承認をめざす
③末期癌患者の悪液質（QOL）改善
④患者血清の*ex vivo*処理による細胞培養用自己血清の調製

図 1 免疫細胞療法から体外循環治療へ—免疫抑制物質吸着繊維カラムの開発目的と用途—
免疫抑制物質（TGF-β，VEGF，IL-6，IAP など）吸着性極細繊維カラムを用いた，血漿交換を伴わない，安全で，簡便で，繰り返し施行可能な癌体外循環治療技術の開発

着効率にも優れることを明らかにした。また，この吸着剤を充填したミニカラムで担癌ラットを1回体外循環治療するだけで，腫瘍の増殖抑制と生存期間の延長効果が得られることも明らかにしている。

本体外循環治療カラムの実際の臨床上の用途としては図1に示したごとく種々考えられるが，当初の開発目的としての免疫細胞療法との連動，そして特に TGF-β が病態の進展に強く関与するスキルス胃癌や膵癌などの難治性癌を対象とし，化学療法との併用による癌治療用医療機材としての承認をめざしている。

1970～1980 年代にかけて，同じく血中の免疫抑制因子の除去を目的として国内外で血漿交換療法が盛んに試みられ一定の成果が報告されたが[47,48]，われわれが開発中のカラムは，血漿交換に伴う種々の副作用を回避し癌治療において初めて血液吸着療法の形での治療体系を確立していくことに特色と独創性がある。また新たなる創薬を必要とせず，繊維加工技術のみで癌治療用医療機材を開発して

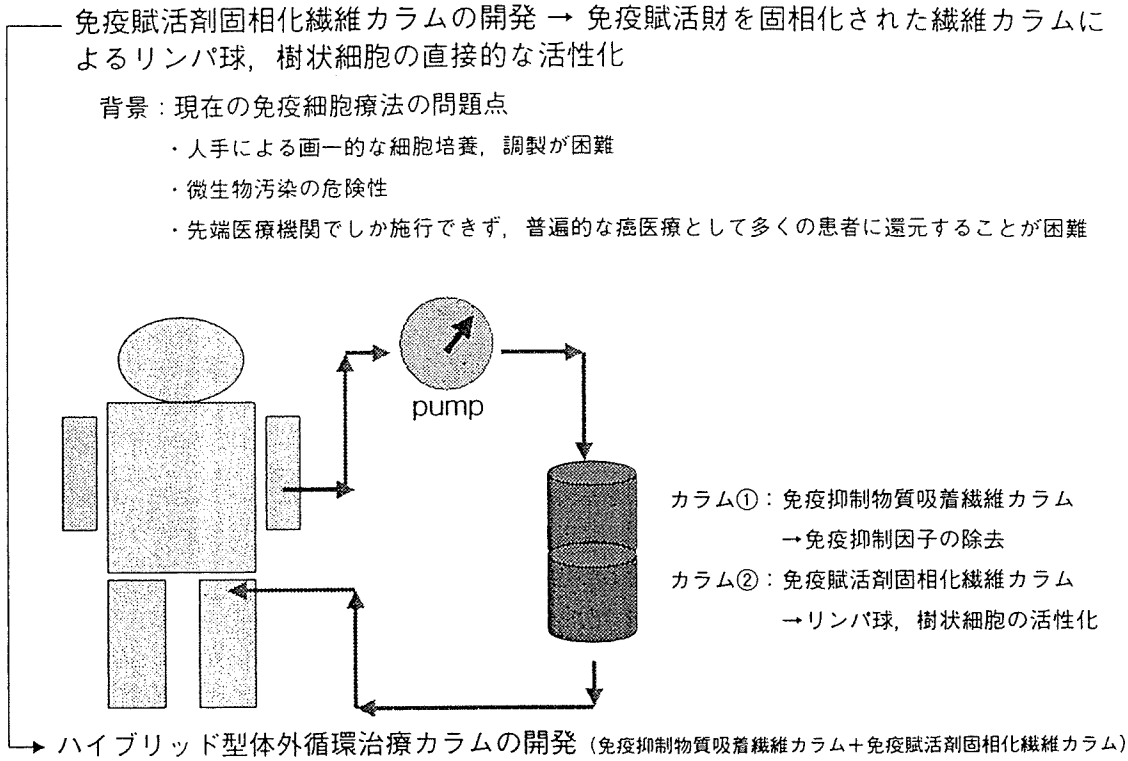


図 2　癌治療用ハイブリッド型体外循環繊維カラムの開発

いくために，承認へ向けた時間の短縮が期待される。

最後に，*in vitro* での細胞処理を伴う現在の免疫細胞療法の多くの問題点を解決するため，2004年からある種の免疫賦活剤を固相化した極細繊維カラムの開発研究を開始している。流血中のリンパ球やDCなどの免疫担当細胞を *ex vivo* に取りだすことなく，体外循環で直に活性化することが目標である。最終的には先の免疫抑制物質吸着繊維カラムと融合させて，完全閉鎖系体外循環による癌免疫細胞療法を構築していきたいと考えている（図2）。

おわりに

ヒト樹状細胞の *in vitro* 誘導と数々の腫瘍拒絶抗原の解明を契機に，受動免疫療法から能動免疫療法へと大きくシフトした固形癌の免疫細胞療法は，再び大きな転換期を迎えつつある。免疫療法としてのsurrogate endpointを充足すれば評価される時代はすでに終わっており，化学療法と共通したprimary endpointである確たる腫瘍の縮小効果と生存期間の延長効果が求められる時代である[49,50]。

化学療法剤の開発研究も，従来のcytotoxic drugからmolecular targeting drug（分子標的治療薬）への開発へと明らかに移行しつつある。これは免疫細胞療法における過去の非特異的から近年の腫瘍特異的治療法の開発の流れと同一であり，将来は免疫療法と化学療法の明瞭な区分がなくなり“癌分子標的治療”という名の下に集約される可能性がある。いずれにしても，今後の癌治療に普遍性，安全性，経済性とともに患者のQOLを重視した簡便性が従来よりいっそう強く求められていくのは間違いない。免疫細胞療法がtranslational researchの枠から抜けだし真の癌治療として今後市民権を得ていくには，少なくとも現在開発されている数々の分子標的治療薬に匹敵する治療成績を，客観的評価に耐え得る臨床試験で証明していかなくてはならない。その道のりは相当険しいと予想されるが，今後の研究の進捗に期待したい。

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と寺本和雄氏との共同研究である。

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担癌ラットにおける免疫抑制物質吸着繊維カラムの細胞性免疫能増強効果

岩本 在弘[*1] 上田 祐二[*1] 寺本 和雄[*2] 島垣 昌明[*2] 山本 芳樹[*1]
伊藤 剛[*1] 清水 健[*1] 塩崎 敦[*1] 玉井 秀政[*1] 山岸 久一[*1]

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The Effects of Direct Hemoperfusion Using the Filtration Column Filled with the Adsorption Fiber for Immunosuppressive Substances on Cell-Mediated Immunity in Tumor-Bearing Rats: Arihiro Iwamoto[*1], Yuji Ueda[*1], Kazuo Teramoto[*2], Masaaki Shimagaki[*2], Yoshiki Yamamoto[*1], Tsuyoshi Itoh[*1], Takeshi Shimizu[*1], Atsushi Shiozaki[*1], Hidemasa Tamai[*1] and Hisakazu Yamagishi[*1] ([*1]*Dept. of Surgery, Division of Digestive Surgery, Kyoto Prefectural University of Medicine, [*2]Specialty Material Research Labs. Toray Industries, Inc. Shiga Plant*)

Summary

The patients with advanced cancer often lose their anti-tumor immune responses due to the increase of some immunosuppressive substances in the blood, such as cytokines and proteins derived from cancer cells or immune cells. We developed the adsorption fiber in transforming growth factor (TGF)-β for the treatment to remove immunosuppressive substances and investigated the effects of direct hemoperfusion using the filtration column filled with this adsorption fiber in tumor-bearing rats. On day 0, KDH-8 tumor cells (1×10^6 cells/rat) were implanted subcutaneously into the back of WKAH/Hkm rats. On day 21 after tumor implantation, the rats underwent the direct hemoperfusion with this filtration column for 60 minutes. On day 28, the rats were sacrificed and the natural killer (NK) activities of their spleen cells were examined. As a result, the rats that underwent this treatment showed a significant increase in their NK activities compared with those of rats who underwent direct hemoperfusion with an empty column or had no treatment. Therefore, we indicated the possibility of a new immunotherapy technique against cancer using a direct hemoperfusion column filled with an adsorption fiber for immunosuppressive substances. Key words: Direct hemoperfusion, Natural killer activity, Cancer immunotherapy, TGF-β

要旨 癌の進行とともに血中には種々の特異的・非特異的免疫抑制物質が増加し，抗腫瘍細胞性免疫能は抑制される。今回，免疫抑制物質吸着繊維カラム（吸着材としてアミノ基含有多孔質極細繊維を使用）を考案し，これを用いた体外循環による，担癌ラットの細胞性免疫増強効果を検討した。免疫抑制物質吸着繊維カラムを回路内に組み込み60分間の体外循環治療を施行し，脾細胞のnatural killer（NK）活性の検討を行った。免疫抑制物質吸着繊維カラムによる体外循環群は空カラム体外循環群，非循環群に比べ有意なNK活性の増強を認めた。担癌宿主血中の免疫抑制物質を除去するこの体外循環治療は，新たな癌免疫療法となる可能性が示唆された。

はじめに

担癌患者の血中には癌の発展に伴い種々の特異的・非特異的免疫抑制物質が増加し，それに伴い抗腫瘍細胞性免疫能は低下する[1]。これらの免疫抑制物質を十分に除去した後に細胞免疫療法あるいは化学療法を行えば，治療効果を増強することが期待できる[2]。1970年代から80年代にかけて進行癌患者に対し血漿交換による免疫抑制物質除去療法が試みられた。その結果一定の治療効果が示されたが，血漿置換液として大量に使用される血液製剤に起因する感染症リスク，有用な血漿成分の破棄，医療コストなど種々の問題から，本療法は癌治療として普及には至らなかった[3]。近年，癌の伸展に伴う免疫抑制動態の詳細が明らかになり，癌患者の血中に増加してくるtransforming growth factor (TGF)-β[4,5]，interleukin (IL)-6，vascular endotherial growth factor（VEGF）などのサ

[*2] 東レ株式会社・機能材料研究所

連絡先: 〒602-8566 京都市上京区河原町広小路上る梶井町465 [*1] 京都府立医科大学・消化器外科
岩本 在弘

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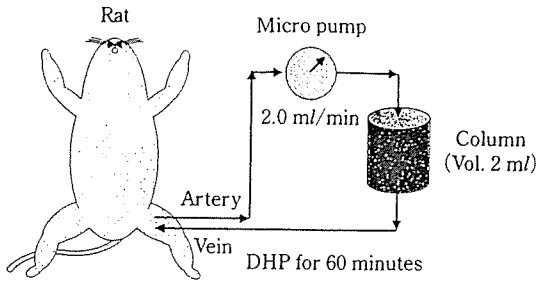

図 1　担癌ラット体外循環治療

イトカインは直に癌の伸展に関与するのみならず，細胞性免疫能を抑制することによりさらに癌の伸展を助長する。また，これらのサイトカインは進行癌患者に悪液質をもたらし化学療法の副作用増強にも関与する。今回われわれは，免疫抑制物質の制御を目的として，免疫抑制物質吸着繊維カラムを用いた血漿交換を伴わない癌体外循環治療を考案し，担癌ラットモデルでその細胞性免疫能の増強効果について検討した。

I．材料と方法

1．担癌ラットモデル

10〜12 週齢の雄 WKAH/Hkm ラット背部皮下に 2×10^6 個の 4-ジメチルアミノアゾベンゼン誘発肝癌 KDH-8 細胞（TGF-β 産生腫瘍）を接種し，担癌ラットを作製した。

2．体外循環治療

吸着材として TGF-β の吸着除去に優れたアミノ基含有多孔質極細繊維状吸着材を充填した容量 2 cc のミニカラムを作製し[6]，担癌 3 週目に全身麻酔下にラットの大腿動脈脱血，大腿静脈返血によるミニカラムを回路内に組み込んだ体外循環を毎分 2 m*l*/min の循環速度で 60 分間施行した（図 1）。

3．Natural killer（NK）活性の測定

免疫抑制物質吸着繊維カラムによる体外循環群，非循環群，空カラム体外循環群の 3 群の担癌ラット脾細胞を用いた NK 活性の比較検討を行った。NK 活性は YAC-1 標的細胞の細胞死をユーロピウム遊離法で測定した[7]。

4．腫瘍増殖とラット生存期間の検討

担癌 2 週目のラットに 2. と同様の体外循環を施行し，その後の腫瘍容積と生存期間を計測した。

II．結　　果

1．NK 活性増強効果

4 時間の短時間 assay，16 時間の長時間 assay ともに体外循環治療群が非循環群，空カラム循環群に比べ NK 活性値の有意な上昇（$p<0.05$）を認めた（図 2，3）。

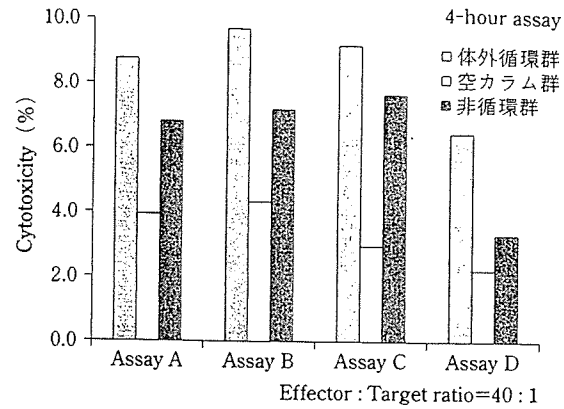

図 2　体外循環による NK 活性増強効果（1）

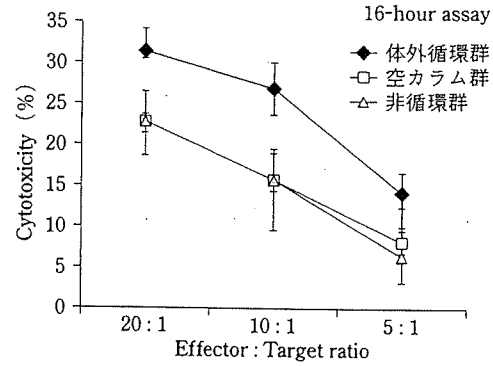

図 3　体外循環による NK 活性増強効果（2）

2．腫瘍増殖抑制効果と生存期間延長効果

免疫抑制物質吸着繊維カラムを用いた体外循環群では腫瘍の縮小を認めたラットもあり，有意な腫瘍増殖抑制（$p<0.05$）と生存期間の延長（$p<0.05$）を認めた（データ非提示）。

III．考　　察

TGF-β は抗腫瘍免疫誘導のあらゆる段階，機構を阻害することが明らかにされている[1]。われわれは新たなる創薬を伴わず，繊維加工技術のみで TGF-β 吸着繊維を開発したが[6]，本体外循環治療は NK 活性を中心とした細胞性免疫能の増強効果を介して，抗腫瘍効果を発現する可能性が示唆された。将来の臨床応用を前提とし，本治療技術は血漿交換療法と異なり，安全に，安価に，繰り返し施行可能なことが利点である。今後，外科治療，化学療法，細胞免疫療法との組み合わせにより，進行，再発癌に対するより有効な集学的治療システムを構築できる可能性がある。

本論文の要旨は第 26 回癌免疫外科研究会において発表された。

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A Newly Identified MAGE-3-Derived, HLA-A24-Restricted Peptide Is Naturally Processed and Presented as a CTL Epitope on MAGE-3-Expressing Gastrointestinal Cancer Cells

Naoto Miyagawa Koji Kono Kousaku Mimura Hideo Omata
Hidemitsu Sugai Hideki Fujii

First Department of Surgery, University of Yamanashi, Yamanashi, Japan

Key Words
MAGE-3 · HLA-A24 · Gastrointestinal tract cancer · Epitope

Abstract
Purpose: In order to broaden the possibility for anti-MAGE-3 immune targeting, it is important to identify HLA-A24-restricted epitopes derived from MAGE-3, since HLA-A24 is one of the most common alleles in Japanese and Asian people. In the present study, we defined a new MAGE-3 derived, HLA-A24-binding peptide presented as a CTL epitope on gastrointestinal cancer cells. ***Materials and Methods:*** A panel of MAGE-3-derived peptides (9mer and 10mer) with the HLA-A24-binding motif was selected, and identification of MAGE-3-derived, HLA-A24-restricted CTL epitopes was performed by a reverse immunology approach. To induce MAGE-3-peptide specific CTLs, PBMCs were repeatedly stimulated with monocyte-derived, mature DCs pulsed with the peptides. Subsequent peptide-induced T cells were tested for their specificities by ELISPOT, tetramer and cytotoxic assay. CTL clones were then obtained from the CTL line by limiting dilution. ***Results:*** The peptide-inducing CTLs revealed that MAGE-3(113)-peptide was reacted as a CTL epitope in a HLA-A24-restricted fashion, confirmed by ELISPOT and cytotoxic assays. In addition, the MAGE-3(113)-specific CTL clones, confirmed by tetramer assay, showed that the MAGE-3(113) epitope is naturally processed and presented as the CTL epitope on MAGE-3-expressing gastrointestinal cancer cells by evaluating the cold target inhibition assays. ***Conclusion:*** The newly identified MAGE-3(113)-peptide epitope is naturally processed and presented as the CTL epitope on MAGE-3-expressing gastrointestinal cancer cells, indicating that anti-MAGE-3 immune targeting with the MAGE-3(113) peptide is a promising approach for treatment.

Introduction

It is now well established that small peptide epitopes, when bound to MHC class I molecules on the surfaces of tumor cells, can be recognized as antigens (Ags) by cytotoxic T lymphocytes (CTLs). Tumor-specific CTLs, adoptively transferred or activated in vivo by tumor-associated CTL epitopes, have therapeutic activity and can induce regression of established tumors or micrometastases [1, 2]. The development of immunotherapeutic methods to treat cancer is critically dependent on the identification of tumor-associated Ags.

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Koji Kono, MD, PhD
First Department of Surgery, University of Yamanashi
1110 Tamaho, Yamanashi, 409-3898 (Japan)
Tel. +81 552 73 6751, Fax +81 552 73 9574
E-Mail kojikono@yamanashi.ac.jp

Table 1. Sequence of MAGE-3-derived peptides with the HLA-A2402-binding motif

	Position[a] (mer)	Sequence
pep76	76 (9)	NYPLWSQSY
pep97	97 (9)	TFPDLESEF
pep113	113 (9)	VAELVHFLL
pep142	142 (9)	NWQYFFPVI
pep150	150 (9)	IFSKASSSL
pep175	175 (10)	LYIFATCLGL

[a] Residue number of the first position of the peptide in relation to the sequence of the entire MAGE-3 gene product.

As an alternative to the genetic and biochemical approaches for identifying tumor-associated CTL epitopes, a reverse immunology method has been developed [3–5]. In this method, MHC class I-binding epitopes are identified and their corresponding synthetic peptides are tested for their capacity to induce peptide- and tumor-specific CTLs derived from healthy individuals or cancer patients. This approach has recently been used for the definition of several new CTL epitopes in different melanoma Ags [3–5].

Cancer-testis antigens (CTA) such as MAGE, BAGE, GAGE and NY-ESO-1 are recognized as attractive targets due to their unique expressions in malignant tumors with the exception of male germ lines, which do not carry HLA molecules [6]. A number of antigenic peptides encoded by MAGE-3 or NY-ESO-1 in context with various HLA molecules have been identified [7–9]. Moreover, several examples from clinical trials suggest that MAGE-3-derived peptides can generate specific T cell responses in patients with melanomas [10] and gastrointestinal (GI) tract cancers [11], in which tumor regression was reported in some metastatic melanoma patients [10].

GI tract cancers are most common in Japan today. Despite the aggressive treatment modalities such as surgical resection with extensive lymphadenectomy and surgery combined with chemo-radiotherapy [12, 13], the control of GI tract cancer at the advanced stage remains difficult. Therefore, immunoadjuvant therapy such as the utilization of antitumor T cells or antibodies against tumor antigens is extremely appealing. It has been reported that MAGE-3 was expressed in 57% of esophageal carcinomas [14] and 38% of gastric carcinomas [15]. HLA-A24 is one of the most common alleles in Japanese people and is shared by more than 60% of the population [16]. Therefore, in order to broaden the possibility for anti-MAGE-3 immune targeting in GI tract cancer, it is important to identify HLA-A24-restricted peptide epitopes derived from MAGE-3. Furthermore, the expression of MAGE-3 is heterogeneous among patients and between individual tumor lesions [17], suggesting that immunotherapy targeting multiple antigenic epitopes is more desirable than a single epitope.

In the present study, we identified a new HLA-A24-restricted, MAGE-3-derived CTL epitope, which is naturally processed and presented as the CTL epitope on MAGE-3-expressing GI tract cancer cells.

Material and Methods

Cell Lines

MKN-7 (HLA-A26 gastric cancer), KATO III (HLA-A2402 gastric cancer), MRKnu-1 (HLA-A2402 breast cancer) and WiDr (HLA-A2402 colon cancer) were obtained from the IBL cell bank (Gunma, Japan). TE-4 (HLA-A0207 esophageal cancer) was a kind gift from Dr. Nishimura (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University, Japan). K562 was obtained from ATCC (Rockville, Md., USA). TISI cells were from a human B lymphoblastoid cell line expressing HLA-A24. These cell lines were kept in RPMI 1640 with 5% FCS, 50 U/ml penicillin and 2 m*M* *L*-glutamine.

Peptide Synthesis

MAGE-3-derived peptides were identified on the basis of the presence of an HLA-A2402-binding motif using a computer program which takes into account the effect of both primary and secondary anchor residues (table 1). Peptides were synthesized by standard solid phase methods and purified by HPLC. HIV peptide with HLA-A2402-binding capacity (ILKEPVHGV) was used as a negative control peptide.

Preparation of DCs

DCs were generated from PBMC in HLA-A24(+) healthy donors and gastric cancer patients. Briefly, PBMC were separated from peripheral blood by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and monocytes were enriched by adherence to a plastic tissue culture flask (Corning, N.Y., USA) for 90 min at 37°C. Adherent cells were cultured with 1,000 U/ml of granulocyte macrophage colony-stimulating factor (GM-CSF, Peprotech EC Ltd., London, UK) and 1,000 U/ml of IL-4 (Peprotech EC Ltd.) in X-VIVO (Life Technologies Inc., Gaithersburg, Md., USA). On day 5, DCs were matured with recombinant CD40 ligand (R&D System, Inc., Minneapolis, Minn., USA) and were used as mature DCs on day 7.

Generation of MAGE-3 Peptide Specific CTL Lines and CTL Clones

Mature DCs were pulsed with MAGE-3 peptides (20 μg/ml) in the presence of β_2-microglobulin (3 μg/ml) for 60 min at 37°C. Then, these peptide-loaded mature DCs were co-incubated with autologous PBMCs in a ratio of 1:10 in a 12-well plate in X-VIVO with 1% autologous serum and 100 IU/ml of IL-2 (Peprotech EC).

Subsequent cultured cells were re-stimulated with these peptide-loaded, irradiated (25Gy) mature DCs every 14 days. After 4 stimulations, cultured CTL lines were tested for reactivity with ELISPOT analysis and cytotoxic assay.

CTL clones were then obtained from the CTL lines by limiting dilution. Briefly, the CTLs were isolated in 96-well U-bottom plates in X-VIVO with irradiated allogeneic PBMC (5×10^4 cells/well) from two different donors in the presence of MAGE-3 peptide (20 μg/ml) and 100 IU/ml of IL-2. The CTL clones were expanded with irradiated allogeneic PBMC, MAGE-3 peptide, and 100 IU/ml of IL-2.

ELISPOT Analysis

MAGE-3 specific response was determined by IFN-γ enzyme-linked immunospot (ELISPOT) analysis. ELISPOT analysis was performed with the Mabtech assay system (Nacka, Sweden). After 96-well plates with nitrocellulose membranes (Millipor) were pre-coated with a primary anti-IFN-γ antibody (1D1K) for 24 h, the plates were pre-reacted with AIM-V containing 1% human serum albumin. Target cells ($2 \times 3\ 10^4$/well) and CTL ($2 \times 3\ 10^3$/well) were incubated in 200 μl of AIM-V for 24 h in triplicate. Thereafter, a biotinylated secondary anti-IFN-γ antibody (7-B6-1) was added for 2 h, and then the plates were incubated with streptavidin-alkaline phosphatase reagent and stained with NBT and BCIP (Gibco).

Cytotoxic Assay

A standard 4-hour ^{51}Cr release assay was performed. To assess the peptide-specificity of CTL, TISI cells were pulsed with MAGE-3 peptide for 16 h at 37°C. Thereafter, peptide-pulsed TISI cells were washed and subjected to cytotoxic assay as a target. After the target cells were labelled with 100 μCi ^{51}Cr for 60 min, target cells (5×10^3/well) and effector cells at various effector/target ratios were co-incubated in 200 μl of X-VIVO in a 96-well U-bottom plate in triplicate for 4 h at 37°C. Subsequently, cold target inhibition was done using nonradiolabeled TISI loaded with MAGE-3 peptide or with the irrelevant peptide (HIV peptide) used as a negative control at various hot/cold target ratios. Supernatants were harvested and radioactivity was determined using a gamma counter. The percentage of ^{51}Cr release was calculated according to the following formula: % lysis = 100× (experimental release-spontaneous release)/(maximum release-spontaneous release).

To perform the inhibition of cytotoxicity with anti-HLA class I mAb (W6/32), target cells were preincubated with mAb using a 1/10 dilution for 1 h at room temperature before the cytotoxic assay.

Tetramer Assay

To evaluate the specificity of the MAGE-3-reacted CTL clones, FITC-labeled anti-CD8 (MBL, Nagoya, Japan), PE-labeled HLA-A2402-MAGE-3(113)-tetramer (NH_2-OOH) or PE-labeled HLA-A2402-MAGE-3(195)-tetramer (NH_2-COOH)(MBL, Nagoya, Japan) was used for immunostaining, according to the manufacturer's recommendations.

RT-PCR for MAGE-3

Total RNA was extracted from tumor cell lines according to the standard protocol with an RNeasy Minikit (Qiagen K.K., Tokyo, Japan). One microgram of total RNA was added to the reaction mixture using the One-Step RT-PCR Kit (Qiagen), and was amplified in a GeneAmp PCR System 9700 (Applied Biosystems, Calif., USA) in a total volume of 50 μl, which contained 5 × one-step RT-PCR buffer 10 μl, dNTPMix 2.0 μl, one-step RT-PCR Enzyme Mix 2.0 μl and 0.6 μ*M* of each specific primer. Specific primers were designed as follows: *MAGE-3* primers, 5'>TGGAGGACCAGAGGCCCCC<3' (forward) and 5'>GGACGATTATCAGGAGGCCTGC<3' (reverse); β-actin primers, 5'>CTACAATGAGCTGCGTGTGC<3' (forward) and 5'>CGGTGAGGATCTTCATGAGG<3' (reverse).

RT reactions were carried out according to the manufacturer's recommendations with 1 cycle of 30 min at 52°C for reverse transcription and 15 min at 95°C for the initial PCR activation step. For *MAGE-3* PCR, the cycling conditions were as follows: 35 cycles of 1 min at 94°C for denaturation, 1 min at 73°C for annealing and 2 min at 72°C for elongation. The amplified product (725 bp for *MAGE-3* and 314 bp for β-actin) was electrophoresed on a 1.2% agarose gel (Ultra Pure, Gibco BRL, New York, N.Y., USA) and equilibrated in TAE (40 m*M* Tris-acetate, 2 m*M* EDTA). Ethidium bromide (0.5 μg/ml) was added to the agarose-TAE gels with TAE electrophoresis buffers to visualize the amplified DNA fragments and these were photographed using Polaroid film 667 under UV light.

Results

Generation of MAGE-3-Derived, HLA-A24-Binding Peptide-Specific CTL Lines

The sequences of MAGE-3 molecules for the probable HLA-A24-binding peptide were screened using a computer program (table 1). With these peptides, we generated 6 different peptide-inducing T cell lines from HLA-A24 (+) healthy donors (n = 7) using mature DCs pulsed with each peptide. Then, the T cell lines were each tested for their specificities against cognate peptides, which were used for each induction, with ELISPOT analysis. Representative data of the reactivities of peptide-inducing T cell lines is shown in figure 1. As a result, two peptide-inducing T cell lines (CTL113 and CTL142) out of 6 T cell lines significantly recognized TISI targets pulsed with each cognate peptide. These observations were confirmed in 6 of 7 different healthy donors.

MAGE-3(113) Peptide-Specific CTL Lines Can Specifically Recognize HLA-A24 Tumor Cell Lines Expressing MAGE-3

The expression of MAGE-3 mRNA on tumor cell lines was analyzed by RT-PCR (fig. 2). CTL113 and CTL142 were tested against MAGE-3-expressing tumor cell lines with ELISPOT analysis (fig. 3). Only CTL113 recognized HLA-A24-positive tumor cell lines expressing MAGE-3 (MRK-nu-1 and WiDr), but not HLA-A24-negative, MAGE-3-positive MKN-7 or TE-4 (fig. 3a), while CTL142 did not react with any tumors (fig. 3b).

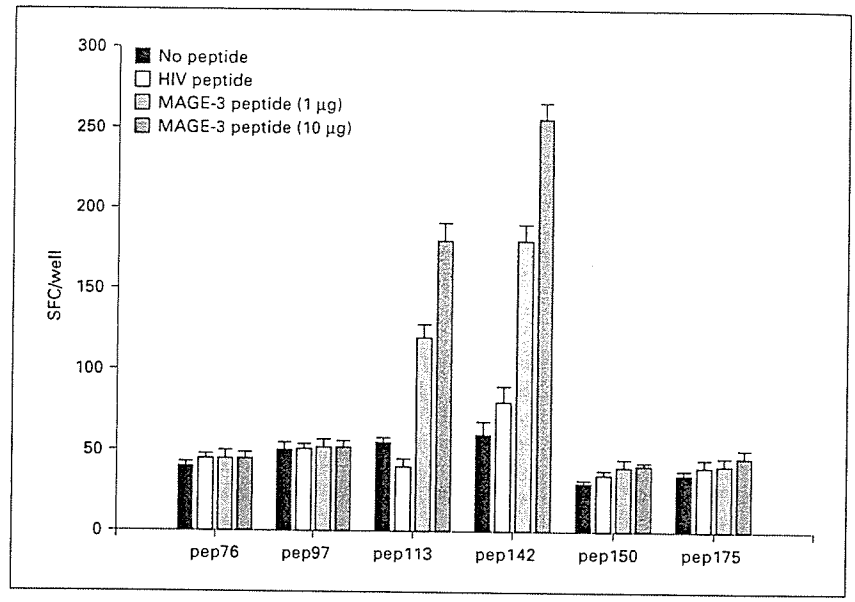

Fig. 1. Reactivities of MAGE-3-peptide-inducing T cell lines evaluated by ELISPOT assay. Peptide inducing CTL lines were generated from PBMCs in HLA-A24(+) healthy donors (n = 7) by repeated stimulation with matured DCs pulsed with the panel of MAGE-3 derived, HLA-A24-binding peptides. After four rounds of stimulation, the CTL lines were tested for their specificities against TISI pulsed with MAGE-3 peptides (1 or 10 μg/ml) or HLA-A24-binding HIV peptides (10 μg/ml) as a control by the ELISPOT assay. Representative data from 7 different donors is shown. SFC = Spot-forming cell.

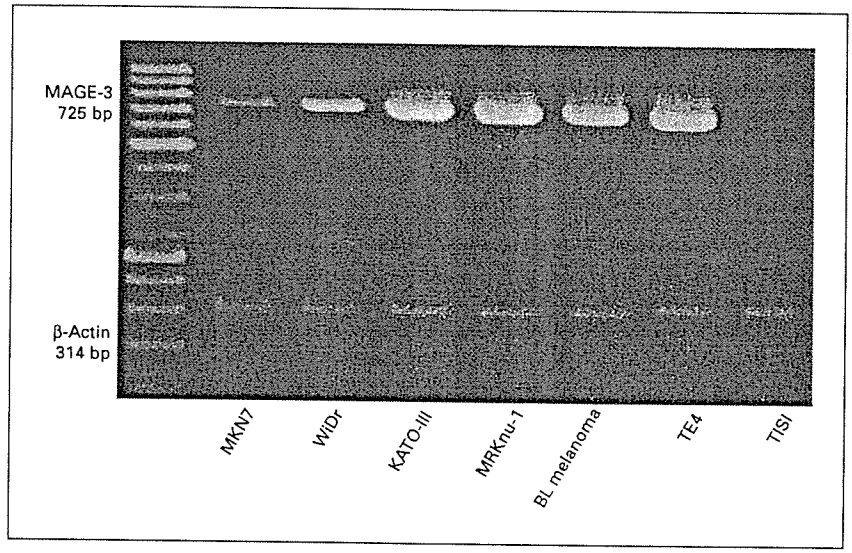

Fig. 2. The expression of MAGE-3 mRNA on tumor cell lines analyzed by RT-PCR. MAGE-3-specific bands by RT-PCR were confirmed for MKN7, WiDr, KATO-III, MRKnu-1 and TE4 cell lines. BL melanoma, a positive control for MAGE-3, and TISI, a negative control for MAGE-3, were included.

To further confirm the reactivity of CTL113, it was tested against several targets in a cytotoxic assay. CTL113 lysed MAGE-3(+), HLA-A24(+) WiDr and the cytotoxicity was inhibited by treatment with anti-MHC class I mAb (fig. 4a). Furthermore, CTL113 lysed TISI pulsed with MAGE-3(113) peptide, but did not lyse TISI pulsed with HIV control peptide (fig. 4b). These results indicate that MAGE-3(113) peptide-inducing CTL recognized and lysed MAGE-3-expressing and HLA-A24(+) tumors, specifically.

CTL113 Clones Recognize HLA-A24 Tumor Cell Lines Expressing MAGE-3 and TISI Target Pulsed with MAGE-3(113) Peptides

To further analyze the specificity of the MAGE-3(113) epitope, CTL clones were generated by limiting dilution methods from the CTL113 line. Using the MAGE-3(113)-HLA-A24 tetramer, CTL113 clones were stained positive for the MAGE-3(113)-tetramer, but not for the MAGE-3(195)-tetramer (fig. 5), indicating that CTL113-clones were MAGE-3(113)-epitope specific. The ELISPOT as-

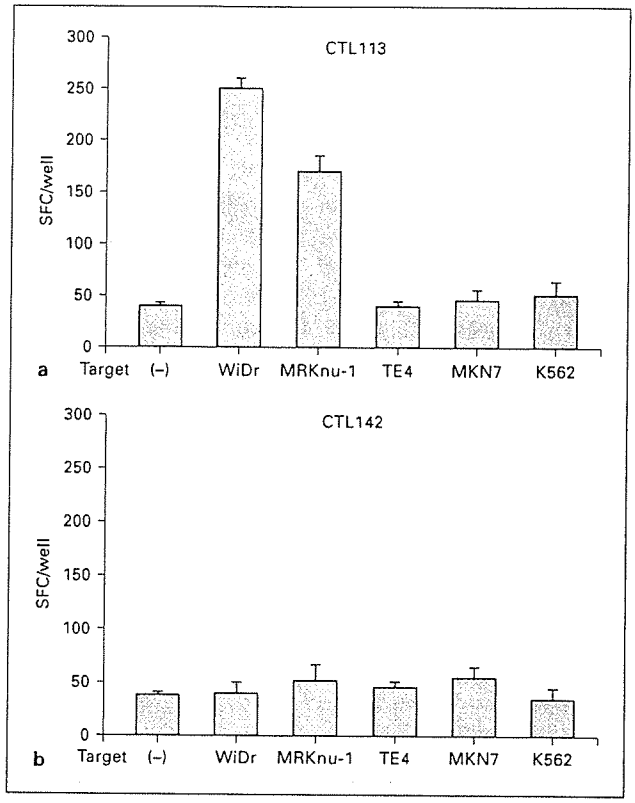

Fig. 3. Reactivities of CTL113 (**a**) and CTL142 (**b**) for MAGE-3-expressing tumor cells evaluated by ELISPOT assay. The reactivities of CTL113 and CTL142 were tested for HLA-A24-positive, MAGE-3-positive WiDr and MRKnu-1 cells or HLA-A24-negative MAGE-3-positive TE4 and MKN7 cells. CTL113 reacted with WiDr and MRKnu-1, but not with TE4 and MLN7, while CTL142 did not react with any MAGE-3-expressing tumor cells.

say indicated that CTL113 clones recognized TISI pulsed with MAGE-3(113) peptides and MAGE-3-expressing, HLA-A24(+) tumors (MRKnu-1 and WiDr), as shown in figure 6.

To further confirm the reactivity of the MAGE-3(113) peptide, various doses of MAGE-3(113) peptides were tested for their capacity to sensitize TISI by the CTL113 clones. As expected, the reactivity of the MAGE-3(113) peptide was dose-dependent (fig. 7).

Then, cold target inhibition assays were performed using non-radiolabeled TISI loaded with the MAGE-3(113) peptide or the irrelevant HLA-A24-binding HIV peptide at various hot /cold target ratios. A significant ((65.2%) at the 1:10 hot to cold ratio) inhibition of the killing for the WiDr mediated by CTL113 clones was observed when non-radiolabeled TISI loaded with the MAGE-3(113) peptide was added, but not TISI loaded with the HIV peptide (fig. 8). Thus, these data indicate that the newly identified MAGE-3(113) peptide is naturally processed and presented as a CTL epitope on MAGE-3 expressing tumors.

Generation of MAGE-3(113)-Specific CTLs from Patients with Gastric Cancers Expressing MAGE-3

To further confirm the reactivity of the MAGE-3(113) epitope in patients, MAGE-3(113)-specific CTLs were generated from HLA-A24(+) patients with gastric cancers expressing MAGE-3 (n = 4) by repeated stimulations with peptide-pulsing mature DCs, and their reactivities were evaluated by ELISPOT analysis. Representative data from two patients showed that MAGE-3(113)-peptide inducing CTLs recognized HLA-A24-positive tumor cell

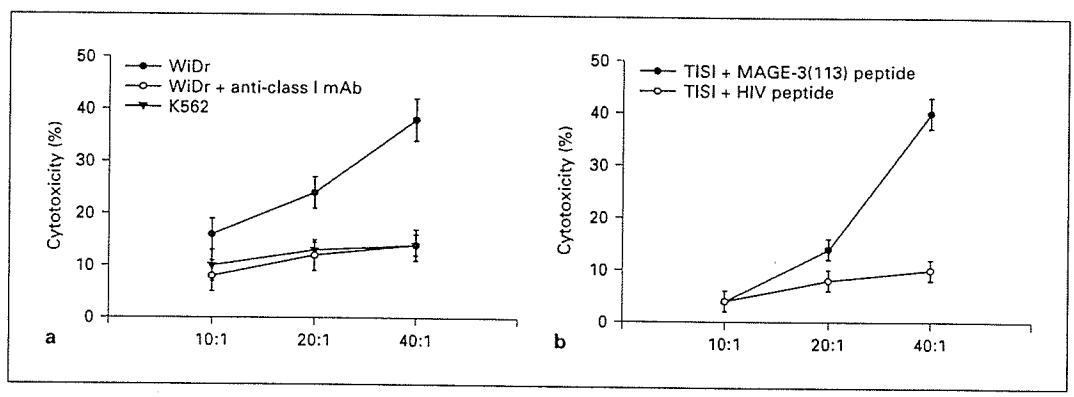

Fig. 4. Cytotoxicity of CTL113 analyzed by 4-hour ^{51}Cr release assay. CTL113 was tested for cytotoxicity against WiDr or K562 (**a**) and TISI pulsed with MAGE-3(113) peptides or HIV control peptides (**b**). The cytotoxicity against WiDr was inhibited by treatment with anti-class I mAbs (**a**).

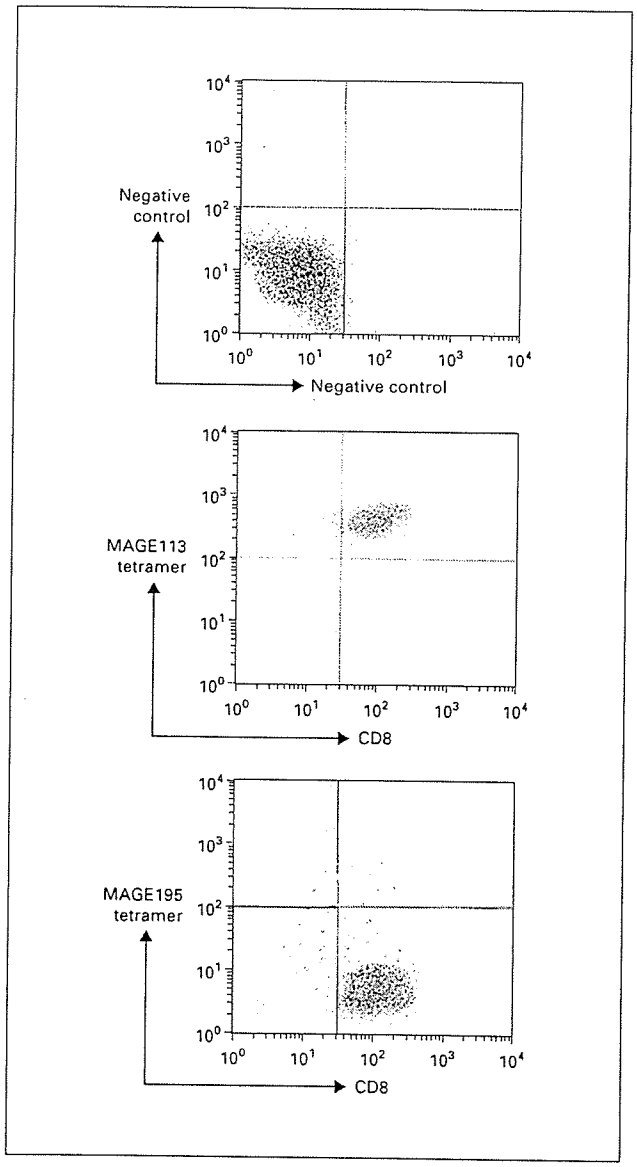

Fig. 5. Tetramer assay for CTL113 clones. CTL clones were generated by limiting dilution methods from the CTL113 line. Using the MAGE-3(113)-HLA-A24 tetramer, the CTL113 clones were stained positive for the MAGE-3(113) tetramer, but not for the MAGE-3(195) tetramer.

lines expressing MAGE-3 (MRK-nu-1 and WiDr), but not HLA-A24-negative, MAGE-3-positive MKN-7 or TE-4 (fig. 9). These results were confirmed in 4 of 4 patients, indicating that MAGE-3(113)-specific CTLs were able to be generated from the cancer patients.

Discussion

In the present study, we screened MAGE-3-derived peptides with the HLA-A24-binding motif using a reverse immunology approach, in order to identify HLA-A24-restricted CTL epitopes derived from MAGE-3. As a result, the newly identified MAGE-3(113) peptide was found to be naturally processed and presented as a CTL epitope on MAGE-3-expressing GI tract cancers.

MAGE-3 was expressed in 38% of gastric cancers [15], 57% of esophageal cancers [14] and 19% of colon cancers [18], in addition to a high proportion of melanomas [6]. These results indicate that anti-MAGE-3 immune targeting is a promising approach for the treatment of GI tract cancers. Until now, several MAGE-3-derived epitopes have been identified, including MAGE-3(195) as HLA-A24-restricted epitope [8], MAGE-3(271) as HLA-A0201-restricted epitope [19] or MAGE-3(168) as HLA-A1-restricted epitope [20]. Among them, it was shown that DCs pulsed with MAGE-3-derived, HLA-A2- or HLA-A24-restricted peptides can induce specific T cell responses in patients with gastric cancers [11]. HLA-A24 is one of the most common alleles in Japanese people and is shared by more than 60% of Japanese gastric cancer patients [16]. In contrast to HLA-A2, the HLA-A24 allele is much less heterogeneous and 99.3% of HLA-A24 is the HLA-A2402 subtype, which is the most common HLA allele in Asian and Japanese people [16]. Thus, it would be desirable to identify additional HLA-A24-restricted immunodominant epitopes derived from MAGE-3, in order to broaden tumor-specific immunotherapy based on MAGE-3.

We showed that MAGE-3(113) peptide-inducing CTLs recognized and lysed MAGE-3-expressing GI tract cancer in an HLA-A24-restricted fashion. Furthermore, the cold target inhibition assay using the MAGE-3(113)-specific CTL clones supported the view that the newly identified MAGE-3(113) peptide is presented as a CTL epitope on MAGE-3 expressing GI cancers. MAGE-3(113) peptide-specific CTLs could be generated from 4 of 4 cancer patients with MAGE-3 expressing tumors in the present study, suggesting that MAGE-3(113) epitope is not tolerated in a cancer-bearing host.

In general, cancer vaccination trials are reported to have a limited clinical response, and there are several problems to be resolved including the surrogate endpoints, trafficking of sufficient numbers of effector cells into the tumor or the presence of regulatory T cells [21]. With regard to MAGE antigens, it remains unclear why most vaccinated patients with MAGE epitopes, including those who displayed tumor regression, had either unde-

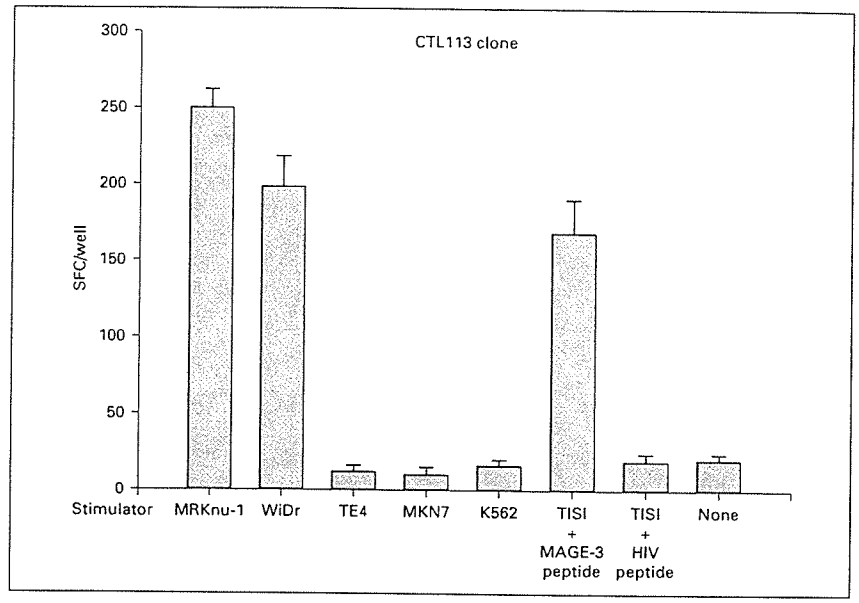

Fig. 6. Reactivities of CTL113 clones for MAGE-3-expressing tumor cells evaluated by ELISPOT assay. The reactivities of CTL113 clones were tested for HLA-A24-positive, MAGE-3-positive WiDr and MRKnu-1 cells or HLA-A24-negative MAGE-3-positive TE4 and MKN7 cells.

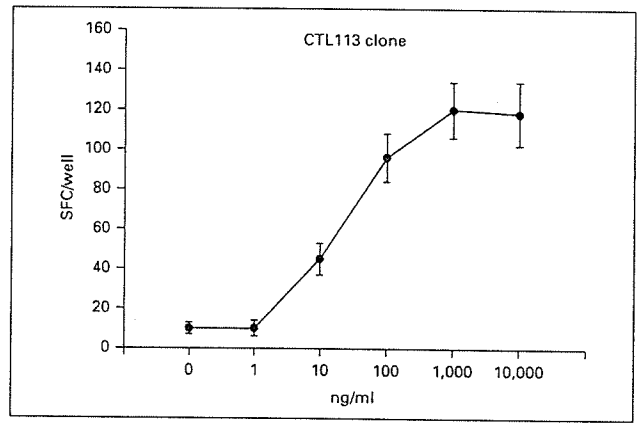

Fig. 7. Dose dependency of MAGE-3(113) epitope. Various doses of MAGE-3(113) peptides were tested for their capacities to sensitize TISI by the CTL113 clones, analyzed by the ELISPOT assay.

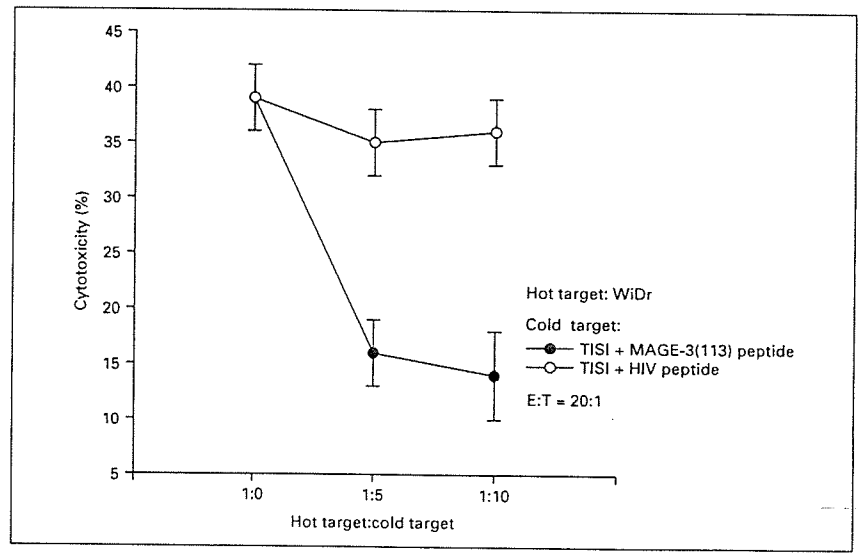

Fig. 8. Cold inhibition assay using CTL113 clones. Cold target inhibition assays were performed using nonradiolabeled TISI loaded with MAGE-3(113) peptides or the irrelevant HLA-A24-binding HIV peptides at various hot/cold target ratios. A significant ((65.2%) at the 1:10 hot to cold ratio) inhibition of the killing for the WiDr mediated by the CTL113 clones was observed when nonradiolabeled TISI loaded with the MAGE-3(113) peptide was added, but not TISI loaded with the HIV peptide.

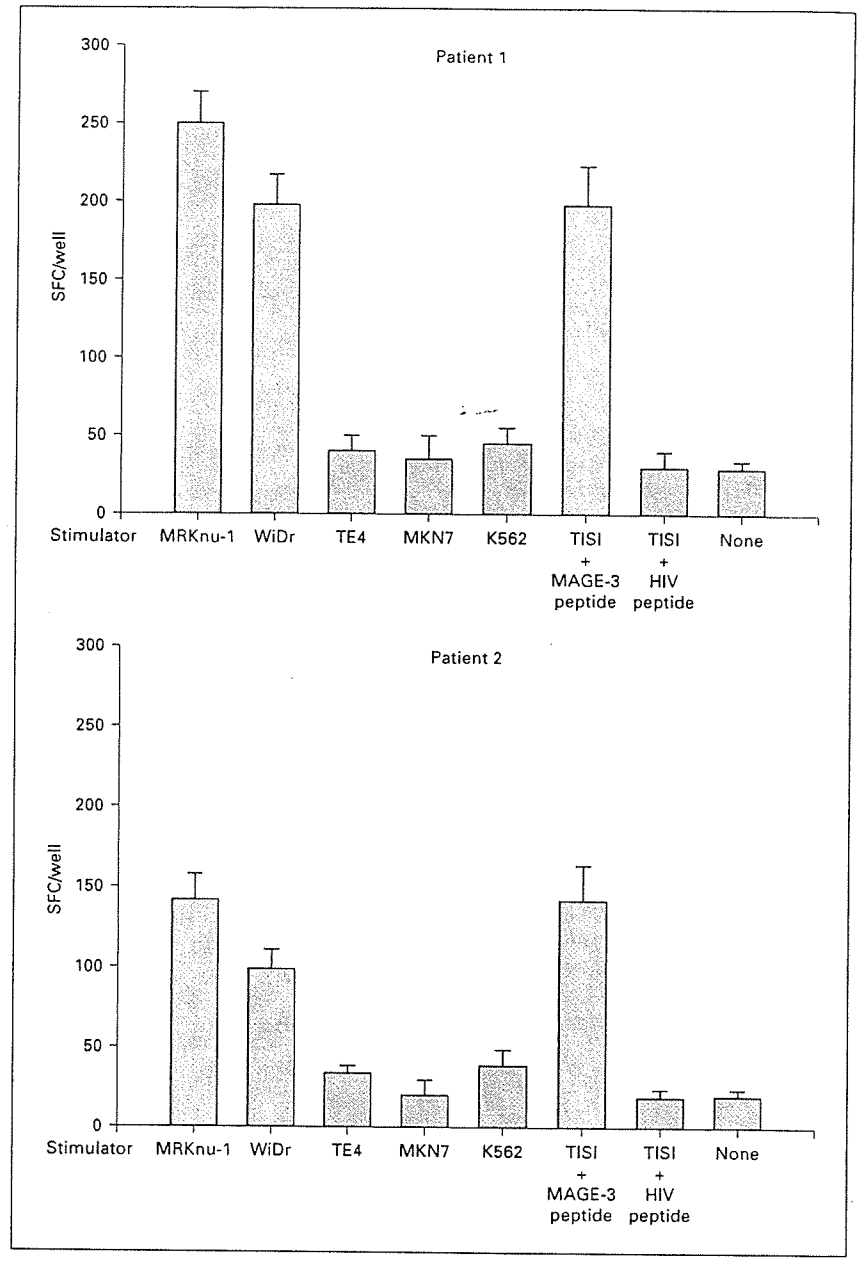

Fig. 9. Reactivities of MAGE-3 peptide-inducing T cell lines generated from cancer patients. Peptide-inducing CTL lines were generated from PBMCs in HLA-A24(+) patients with gastric cancer (n = 4) by repeated stimulation with matured DCs pulsed with the MAGE-3(113) peptide. After four rounds of stimulation, the CTL lines were tested for their specificities against TISI pulsed with MAGE-3 peptide or HLA-A24-positive, MAGE-3-positive WiDr and MRKnu-1 cells or HLA-A24-negative, MAGE-3-positive TE4 and MKN7 cells by the ELISPOT assay. Representative data from 4 different patients are shown.

tectable or very low frequencies of antivaccine CTLs in the peripheral blood [22]. However, it has recently been shown that vaccinated melanoma patients with MAGE had already mounted a strong spontaneous T cell response against several types of tumor antigens before vaccination and had antitumor CTLs at much higher frequencies than those of the antivaccine CTLs after vaccination, suggesting that the anti-vaccine CTLs are not direct effectors for killing the tumors, but that their interaction with the tumor generates conditions enabling the stimulation of large numbers of antitumor CTLs that proceed to destroy the tumor cells [10, 23]. These observations suggested that anti-MAGE targeting by vaccination could stimulate naïve T cells to form new antitumor clo-

notypes against several tumor antigens other than the vaccine antigen.

In the present study, we found that the newly identified MAGE-3(113) epitope is naturally processed and presented as a CTL epitope on MAGE-3 expressing GI tract cancers. Thus, anti-MAGE-immune targeting such as MAGE-3(113) peptide-based vaccination or the adoptive transfer of MAGE-3(113) peptide-inducing CTLs may be a promising approach for the treatment of GI tract cancers as well as melanomas.

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ORIGINAL ARTICLE

Kousaku Mimura · Koji Kono · Scott Southwood
John Fikes · Akihiro Takahashi · Naoto Miyagawa
Hidemitsu Sugai · Hideki Fujii

Substitution analog peptide derived from HER-2 can efficiently induce HER-2-specific, HLA-A24 restricted CTLs

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Abstract In order to broaden the possibility for anti-HER-2/neu (HER-2) immune targeting, it is important to identify HLA-A24 restricted peptide epitopes derived from HER-2, since HLA-A24 is one of the most common alleles in Japanese and Asian people. In the present study, we have screened HER-2-derived, HLA-A24 binding peptides for cytotoxic T lymphocyte (CTL) epitopes. A panel of HER-2-derived peptides with HLA-A24 binding motifs and the corresponding analogs designed to enhance HLA-A24 binding affinity were selected. Identification of HER-2-reactive and HLA-A24 restricted CTL epitopes were performed by a reverse immunology approach. To induce HER-2-reactive and HLA-A24 restricted CTLs, PBMCs from healthy donors were repeatedly stimulated with monocytes-derived, mature DCs pulsed with HER-2 peptide. Subsequent peptide-induced T cells were tested for the specificity by enzyme linked immunospot, cytotoxicity and tetramer assays. CTL clones were then obtained from the CTL lines by limiting dilution. Of the peptides containing HLA-A24 binding motifs, 16 peptides (nine mers) including wild type peptides ($IC_{50} < 1{,}000$ nM) and substituted analog peptides ($IC_{50} < 50$ nM) were selected for the present study. Our studies show that an analog peptide, HER-2(905AA), derived from HER-2(905) could efficiently induce HER-2-reactive and HLA-A24 restricted CTLs. The reactivity of the HER-2(905AA)-induced CTL (CTL905AA) was confirmed by different CTL assays. The CTL905AA clones also were able to lyse HER-2(+), HLA-A24(+) tumor cells and cytotoxicity could be significantly reduced in cold target inhibition assays using cold targets pulsed with the HER-2(905) wild type peptide as well as the inducing HER-2(905AA) analog peptide. A newly identified HER-2(905) peptide epitope is naturally processed and presented as a CTL epitope on HER-2 overexpressing tumor cells, and an MHC anchor-substituted analog, HER-2(905AA), can efficiently induce HER-2-specific, HLA-A24 restricted CTLs.

Keywords Substitution analog · HLA-A24 · HER-2 · Epitope · CTL

K. Mimura · K. Kono (✉) · A. Takahashi · N. Miyagawa
H. Sugai · H. Fujii
First Department of Surgery, University of Yamanashi,
1110 Tamaho, 409-3898 Yamanashi, Japan
E-mail: kojikono@yamanashi.ac.jp
Tel.: +81-552-736751
Fax: +81-552-739574

S. Southwood · J. Fikes
Epimmune, Inc., San Diego, CA 92121, USA

Introduction

It is now well established that small peptide epitopes which bind to MHC class I molecules on the surface of tumor cells can be recognized as antigens (Ags) by cytotoxic T lymphocyte (CTL). Tumor-specific CTL, adoptively transferred or activated in vivo by tumor-associated CTL epitopes, have therapeutic activity and can induce regression of established tumors or micrometastases [23, 27]. The development of immunotherapeutic methods to treat cancer is critically dependent on the identification of tumor-associated Ags. Several immunogenic peptide epitopes, recognized by CTL lines and clones, have been defined from human carcinomas [1, 4, 6, 11, 12, 33].

As an alternative to the genetic and biochemical approach for identifying tumor-associated CTL epitopes, a reverse immunology method has been developed [2, 12, 13, 34]. In this method, predicted MHC class I binding epitopes within a tumor Ag sequence are identified using algorithms of MHC anchor residue motifs and peptides corresponding to these epitopes are synthesized and tested to confirm binding to purified HLA molecules. Peptides demonstrating strong HLA binding affinity are screened further for their capacity to induce peptide- and tumor-specific CTL from healthy individuals or cancer patients. This approach has recently been used for the definition of several new CTL epitopes in different

melanoma Ags [2, 12, 13, 34] as well as tumor Ags expressed on breast, colon and lung adenocarcinomas [15].

The HER-2/neu (HER-2) proto-oncogene encodes a 185-kDa transmembrane glycoprotein that contains an extracellular domain and an intracellular domain with tyrosine-specific kinase activity and has a similarity in structure and sequence to the epidermal growth-factor receptor [5]. HER-2 is amplified and overexpressed in approximately 30% of the human ovarian and breast tumors [29], and in 20% of gastric cancers [10], and is correlated with the stage progression of gastric cancer [19, 30]. In a previous study, we have provided evidence that HER-2-derived peptides are naturally processed as tumor-associated Ags in gastric cancer and can be recognized by tumor-specific, HLA-A2 restricted CTLs [18]. HLA-A2 restricted CTL epitopes derived from HER-2, that are recognized by ovarian [8, 17] and breast [22] cancer-specific CTLs, have previously been defined. Additional HLA-A2 restricted, CTL epitopes derived from HER-2 which can activate CTLs from healthy donors and patients with advanced ovarian carcinoma have also been reported [14, 26]. Based on the above reports, it may be speculated that anti-HER-2 immune targeting may be utilized as a common approach to immunotherapy of a variety of cancers.

HLA-A24 is one of the most common alleles in the Japanese population with more than 60% of this ethnic group expressing this HLA allele [7]. Therefore, in order to broaden the possibility for anti-HER-2 immune targeting, it is important to identify HLA-A24 restricted peptide epitopes derived from HER-2. Furthermore, in this study, we have synthesized analogs of HER-2-derived peptides which are substituted at one or both of the MHC anchor positions of the sequence to enhance HLA binding and immunogenicity. It has been shown that MHC anchor-substituted analogs derived from gp100 can more efficiently induce CTL response than wild type peptide epitopes [25].

In the present study, we describe the identification of a new HLA-A24 restricted, HER-2-derived anchor-substituted analog epitope which efficiently induces CTLs that respond to the native HER-2 wild type peptide epitope as well as to the endogenously processed epitope presented by HLA-A24(+) and HER-2(+) tumor cell lines.

Material and methods

Cell lines

MKN-7 (HER-2+, HLA-A26+ gastric cancer), KATOIII (HER-2+, HLA-A24+ gastric cancer), MRKnu-1 (HER-2+, HLA-A24+ gastric cancer), WiDr (HER-2+, HLA-A24+ colon cancer) and PC-9 (HER-2+, HLA-A24+ lung cancer) were obtained from the IBL cell bank (Gunma, Japan). HCT-15 (HER-2+, HLA-A24+ colon cancer), SKOV 3 (HER-2+, HLA-A3/A11+ ovarian cancer) and K562 (HER-2−, HLA-A24− lymphoma cell) were obtained from ATCC (Rockville, MD). TISI cells are human B-lymphoblastoid cell lines expressing HLA-A24. These cell lines were kept in RPMI 1640 with 5% FCS, 50 U/ml penicillin and 2 mM L-glutamine.

Peptide synthesis

Peptides were either synthesized at Epimmune, Inc. (San Diego, CA), as previously described [28], or, for large epitope libraries, purchased as crude material from Mimotopes (Clayton, Victoria, Australia). Peptides synthesized at Epimmune were purified to >95% homogeneity by reverse-phase HPLC. Purity was determined on an analytical reverse-phase column and the composition was ascertained by amino acid analysis and/or mass spectrometry analysis. In the present study, we have synthesized HER-2-derived peptides with HLA-A24 binding motifs and the corresponding analogs designed to enhance HLA-A24 binding affinity.

HLA-A24 binding assay

The peptide binding assay specific for HLA-A24 molecules has been described previously [16, 21]. Briefly, the assay is based on the inhibition of a radiolabeled standard peptide to detergent solubilized HLA molecules by unlabeled test peptides. The standard peptide, with the sequence AYIDNYNKF, was radiolabeled with ^{125}I by the chloramine T method. HLA-A24 molecules were purified by affinity chromatography from detergent extracts prepared from the EBV-transformed cell line KT3, as previously described [16]. Purified human HLA-A*2402 molecules, at a concentration which bound approximately 10–20% of the total radioactivity (generally between 5 and 15 nM), were incubated with 1–10 nM of the ^{125}I-radiolabeled probe peptide and varying doses of test peptide ranging from 120 μg/ml to 1.2 ng/ml. The binding reaction between HLA molecules, standard peptide and the competing test peptide was carried out in the presence of 1 μM human β_2-microglobulin (Scripps Laboratories, San Diego, CA) and a cocktail of protease inhibitors for 48 h at room temperature. Class I peptide complexes were then separated from the free peptide by gel filtration on TSK2000 columns (Tosohaus, Montgomeryville, AL). Peptide binding was quantified by determining the concentration of peptide required to inhibit the binding of the radiolabeled standard peptide by 50% (IC_{50}%). Peptides were tested in 2–4 independent experiments. The average IC_{50} level of the standard peptide was 6.0 nM.

Preparation of DCs

DCs were generated from PBMC from HLA-A24 healthy donors. Briefly, PBMCs were separated from