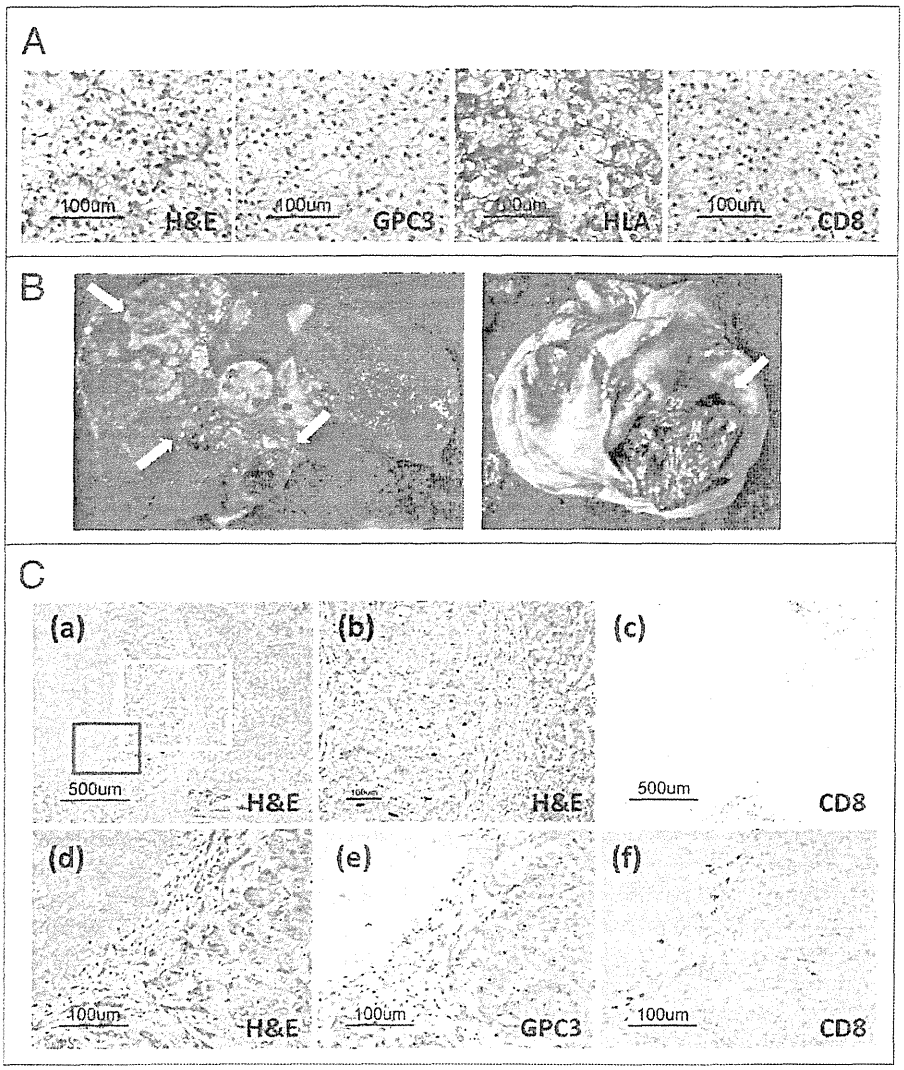

Figure 2. (**A**) Pathological findings of liver biopsy specimens before vaccination. A microscopy image of a hematoxylin and eosin (H&E)-stained section shows well-differentiated hepatocellular carcinoma (HCC). Immunohistochemical staining for GPC3 and HLA class I showed positivity in the cytoplasm and membranes of carcinoma cells, respectively. No CD8-positive T cells were observed in carcinoma tissue before vaccination. (**B**) Macroscopic findings of the liver and heart before formalin fixation at the time of autopsy. Most liver tumors had a necrotic area (arrow). A tumor thrombus occupied most of the right atrium (arrow). (**C**) Pathological findings of the autopsy specimen. (**a**) Microscopic images of H&E-stained sections showing central necrosis of carcinoma tissue, whereas a cirrhotic nodule adjacent to the carcinoma tissue was not necrotic. (**b**) Magnified image of the area enclosed within the white box in (**a**) showing that cancer cells exhibited a morphology (left) different from that of cirrhotic cells (right). (**c**) CD8-positive T cells (brown) infiltrated the carcinoma cells accompanied by necrosis. In contrast, no infiltration of CD8-positive T cells was detected within the cirrhotic nodule. (**d**) Magnified image of the area enclosed within the red box in (**a**) showing necrosis and viable carcinoma cells. (**e**) Positive immunohistochemical GPC3 staining was observed in only the cytoplasm of carcinoma cells. (**f**) CD8-positive T cells infiltrated the necrotic area and carcinoma tissue.

Necrosis was found in the center of each tumor; therefore, the central necrosis caused by ischemia, in addition to CD8-positive T cells attacking tumor cells, may have led to tumor necrosis. Three findings support the hypothesis that tumor necrosis was caused by CD8-positive T cells, as follows: (1) the necrotic changes determined by CT after vaccination, accompanied by clinical laboratory data; this was consistent with an immune response, although no tumor necrosis was evident on the CT before vaccination; (2) no necrosis was evident in the left lobe (no tumors) of the autopsy liver specimen, but it was present in the right liver lobe (tumors present); and (3) CD8-positive T cells infiltrated residual viable tumor cells. The analyses used in this study may contribute to identifying the pathological state after vaccination.

We detected infiltration of CD8-positive T cells into the hepatic tumors, but little infiltration of CD8-positive T cells into

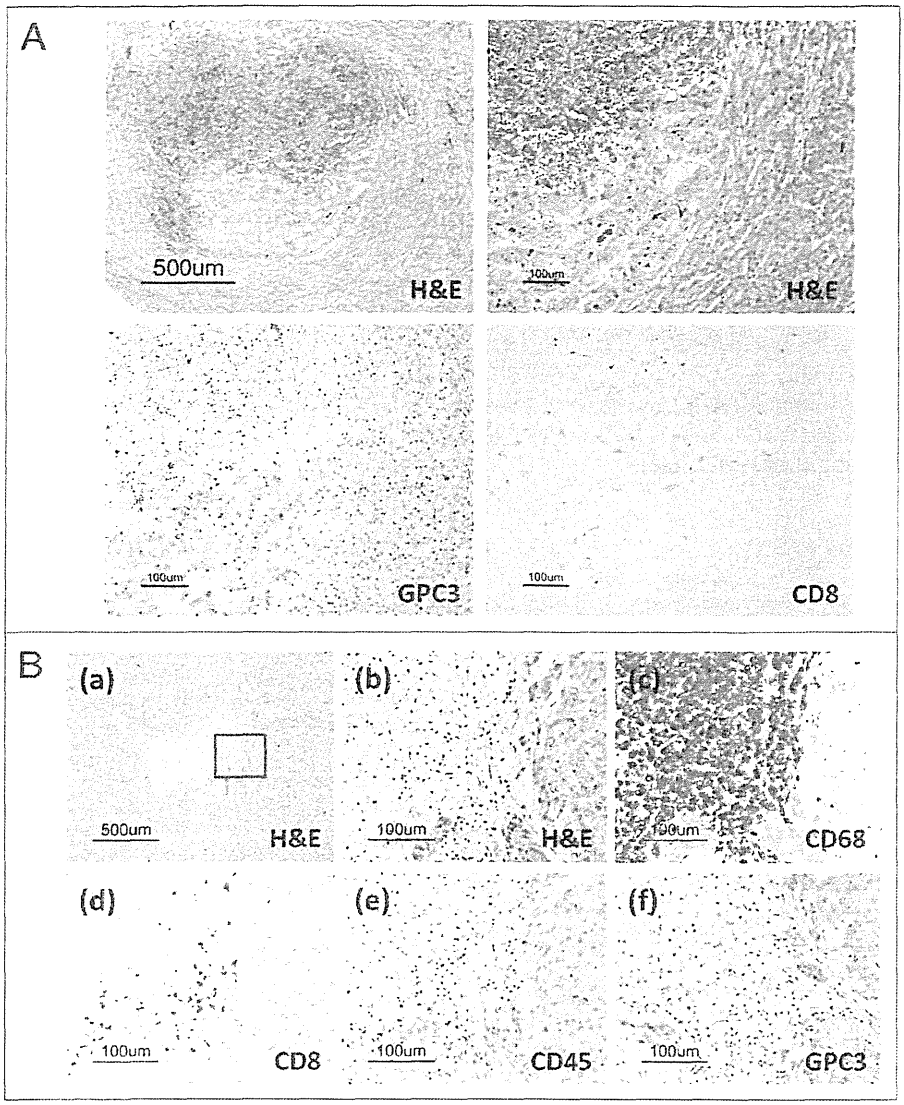

Figure 3. Pathological findings in the autopsy specimen. (**A**) Carcinoma in a cirrhotic nodule. CD8-positive T cells (brown) infiltrated only the carcinoma area, accompanied by necrosis. No infiltration of CD8-positive T cells was detected in the cirrhotic nodule. Only carcinoma cells were GPC3-positive by immunohistochemical staining. (**B**) Necrotic area surrounded by cirrhotic nodules. (**a**) Necrosis was surrounded by viable cirrhotic cells. (**b**) The margin between the necrosis and the cirrhotic nodule. This portion is enclosed by the red box in (**a**). (**c**) CD68-positive macrophages (brown) aggregated in the necrotic area around the cirrhotic nodule. (**d**) CD8-positive T cells (brown) infiltrated the necrotic area but not the cirrhotic nodule. (**e**) CD45-positive lymphocytes infiltrated the necrotic area. Based on the image in (**d**), most of the lymphocytes were CD8-positive T cells. (**f**) Cirrhotic cells did not express GPC3.

the tumor thrombus. This discrepancy may have been caused by the heterogeneity associated with immune-escape mechanisms in tumor cells.

This case report of central necrosis in a patient with HCC might be regarded as spontaneous regression correlated with circulatory failure due to a massive tumor embolism. It was not known whether the tumor necrosis was induced by CTLs, ischemia, or other factors. However, the infiltration of CD8-positive T cells into tumor cells supports immune-related necrosis.

The rate of spontaneous partial regression among patients with HCC is 0.406% compared with the control arm of a randomized clinical trial.[16] In contrast, three of 33 patients who received GPC3 peptide vaccination in the phase I trial had suspicious tumor necrosis on CT scans. In one report, massive infiltration of CD8-positive T cells in the remaining liver tumor and tumor necrosis were identified by histological examination of a biopsy specimen after vaccination.[11] Indeed, on-going clinical trials of the GPC3 peptide vaccine will provide additional information and further demonstrate the antitumor effect.[17,18] Histological results at the estimated time of a strong GPC3-specific CTL response suggest that GPC3 peptide vaccination may be a promising approach to treat HCC.

Materials and Methods

Ex vivo interferon-γ (IFN-γ) enzyme-linked immunospot assay. An ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed to evaluate the antigen-specific CTL response, as described previously.[13] Briefly, peripheral blood (30 mL) was obtained from the patient before the first vaccination and 2 weeks after each vaccination and centrifuged on a Ficoll-Paque gradient. Non-cultured peripheral blood mononuclear cells (PBMCs) (5×10^5/well) were added to plates in the presence of 10 μg/mL peptide antigens and incubated for 20 h. The GPC3 antigen used was the HLA-A2-restricted $GPC3_{144-152}$(FVGEFFTDV) peptide. PBMCs with the HLA-A2-restricted HIV19–27 (TLNAWVKVV) peptide (ProImmune) were used as negative controls. Assays were performed in duplicate.

Immunohistochemical analysis. Immunohistochemical staining with monoclonal antibodies against GPC3 (clone, 1G12; Biomosaics), HLA class I (clone, EMR8/5; Hokudo), CD8 (clone, 1A5; Novocastra), CD45 (cloned 2B11 and PD7/26; Ventana), and CD68 (clone, KP-1; Ventana) was performed according to the manufacturer's protocol.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to declare with regard to this study.

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特集 | 変貌するがん免疫療法

ペプチドカクテルワクチン療法*

大藤和也**
中面哲也**

Key Words：peptide cocktail vaccine, multi-antigen vaccine, glypican-3

はじめに

がん免疫療法が第4のがん治療法として期待されて久しいが，抗体療法と一部のサイトカイン療法を除いては，現在標準治療として確立されたものはない．1991年にBoonらによりメラノーマ抗原MAGE遺伝子が同定され，ヒトの免疫系ががんを異物として認識し，排除しうることに科学的な根拠が与えられた[1]．以降，がん拒絶抗原，エピトープペプチドが多数同定され，さまざまながん種に対するがんワクチン療法の臨床試験が行われた．2004年にはRosenberg博士により，がんワクチンによる奏効率はわずか2.6%であったという悲観的な報告[2]もなされたが，近年では，臨床効果の実証が今後期待できる報告も相次いでいる．2010年には，前立腺がんに対してsipuleucel-T(Provenge®)が，第III相臨床試験[3]の結果を受け，米国食品医薬品局(Food and Drug Administration；FDA)により承認された．さらに，免疫チェックポイント分子であるCTLA-4を阻害するipilibumab(Yervoy®)が悪性黒色腫に対する生存期間の延長を示し[4]2011年に承認された．これらの事項はがん免疫療法の第4のがん治療としての地位の確立に向けての第一歩であるといえる．著者らは新規がん胎児性抗原であるglypican-3(GPC3)を同定し，国立がん研究センター東病院でがんペプチドワクチン療法の臨床試験を行ってきた．その経験をふまえ，本年より本院を中心とする難治性小児がんに対するペプチドカクテルワクチン療法の第I相医師主導治験をスタートさせた．

本稿では，がんペプチドカクテルワクチン療法の意義，展望について著者らの取り組みも交えて概説する．なお，本稿におけるがんペプチドカクテルワクチン療法とは，複数抗原ペプチドを一つのカクテル製剤として用いる治療法とし，複数抗原ペプチドをそれぞれの部位に投与する投与法を複数抗原ペプチドワクチンーノンカクテルと区別して記載する．

細胞傷害性T細胞の抗原認識と腫瘍拒絶

ペプチドワクチンの最大の特徴はその抗原特異性の高さにあるといえる．がん抗原由来エピトープペプチドをワクチンとすることで，正常組織への影響は少なく，有害事象の発現も低いと考えられる．細胞療法等に比べると投与までの過程が煩雑ではなく，外来治療において比較的簡易，安全に投与可能であることが大きな利点である．また比較的安価で，高純度な製剤が製造可能なことも特徴の一つである．このよう

* Peptide cocktail vaccine.

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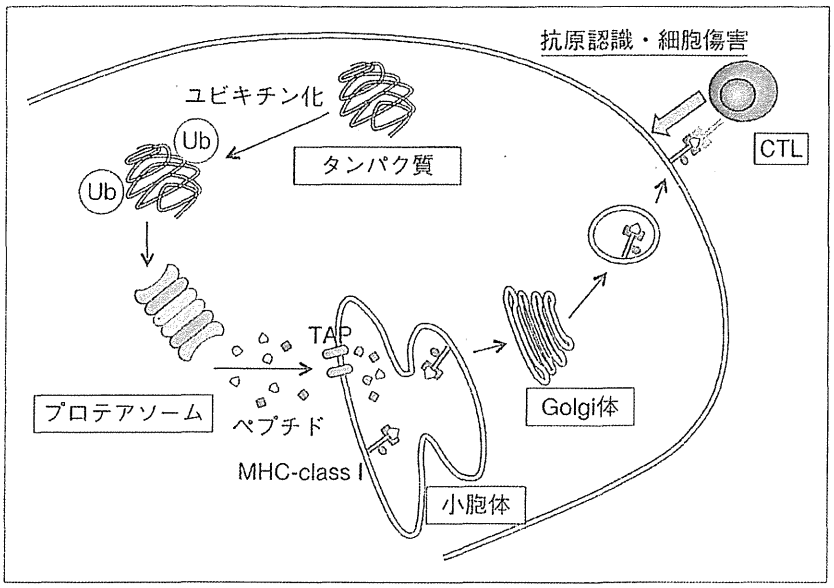

図1　内在性抗原のMHC class I 分子による抗原提示

な背景のもと，本邦ではペプチドワクチン療法を中心にがん免疫療法開発が進められてきた．以下に作用機序を述べるが，がんペプチドワクチン療法はがんに対して免疫機構を介して間接的に作用し効果を発揮する．これは，直接的な効果を有する抗がん剤や低分子化合物とはまったく異なる作用機序を有しており，がんペプチドワクチン開発は，従来の抗がん剤とは異なる考えで進められなくてはならない．

がん細胞拒絶を目的としたワクチン療法は，主に，最終エフェクター細胞と考えられる細胞傷害性T細胞(cytotoxic T lymphocyte；CTL)の誘導を目的としている．CTLはがん細胞膜表面上に提示される，主要組織適合遺伝子複合体(major histocompatibility complex；MHC)と8～10個のアミノ酸で構成されるペプチドの複合体を，抗原として認識する．ワクチンとしてのがん関連抗原の投与には腫瘍ライセート，腫瘍関連蛋白，がん抗原由来ペプチドなどが用いられる．その中でもがん抗原由来ペプチドは，CTLエピトープである8～10個のアミノ酸で構成されており，細胞性免疫誘導を惹起可能な抗原の最小単位である．抗原をワクチンとして投与すると，樹状細胞(dendritic cell；DC)に代表される抗原提示細胞によって抗原提示がなされることによりCTLが誘導される．一方，標的細胞側では，内在性抗原である標的分子はプロテアソームにてペプチド断片化され，小胞体内でMHC分子と結合しゴルジ体を介して膜表面へと運ばれる．この膜表面に提示されたペプチドをCTLが認識し，標的細胞が傷害される(図1)．

上記のような流れでCTLが誘導され標的細胞の認識から拒絶に作用とすると考えられるが，単一のペプチドを投与する場合，誘導されるCTLが認識できるペプチドも基本的に単一であり，複数種類のペプチドを認識できるCTLを誘導することはできない．また，がんワクチン療法の最も重要といえるポイントは，どんなに効率的に細胞傷害性の高いCTLが誘導可能であったとしても，標的細胞側の膜表面に抗原提示がされていなければ細胞傷害活性を示せない点である．一般的に，がん細胞は免疫監視からのさまざまな逃避機構を備えており，一例をあげれば，MHC分子の発現量の不足，標的がん抗原の欠落，接着分子の発現不全，免疫抑制因子の産生，免疫抑制性の微小環境の構築などがある．ワクチンにより誘導されたCTLは，MHC分子の発現低下や，標的抗原の欠落など標的細胞との作用部位に変化を受けた場合，細胞傷害性を示すことはできなくなる．したがって，ペプチドワクチン療法の抗原特異性の高さが同時に弱点ともなりえるといえる．さらに，単一のペプチドによるワク

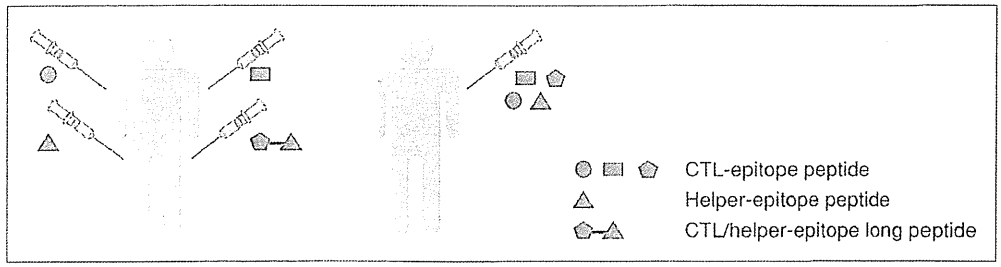

図2　複数のペプチド投与手段
左：それぞれの単一ペプチドを複数か所に投与する．右：複数のペプチドをカクテル製剤として投与する．接種が1か所で済み，多数のペプチドが投与可能となる．

チン療法の場合では，免疫逃避機構による細胞傷害活性の低下は顕著となることが予想され，より有効性の高い新規のペプチドワクチン療法開発が求められる．

がんペプチドカクテルワクチン療法

ペプチドワクチンの効果増強のための一つの手段として，複数のエピトープペプチドをワクチンとして投与する手法が試みられてきた．複数のエピトープペプチドを投与することで，それぞれのペプチド特異的CTLを誘導しようという考えである．さらには，ヘルパーT細胞エピトープを含むワクチンを投与し，より効果増強を狙う試みもなされている[5]．複数個のエピトープの投与を考える場合，単一抗原由来の複数のエピトープを用いる場合と，複数のがん抗原由来ペプチドを用いる場合がある．また，投与法では複数の単一ペプチドを複数か所に投与する(複数抗原ペプチドワクチンーノンカクテル)，あるいはカクテル製剤(がんペプチドカクテルワクチン)として1か所に投与する手段がある(図2)．前者の場合，担がん患者に対し複数か所注射するのは本人への負担も大きく，現実的には投与可能なペプチドの個数も3～4種程度までに限定される．一方で，カクテル製剤とすることで，使用できるペプチド数に理論上の制限はない．

複数抗原に対するペプチドをカクテル製剤，あるいはノンカクテル製剤として投与することによる考えうる利点を，①特異的CTL誘導と②標的細胞の抗原提示の側面からあげる．①まず特異的CTL誘導の側面からみると，現時点でワクチン投与前にペプチド特異的CTLが誘導できるかどうかを予測することはできない．ペプチドを認識するT細胞受容体(T cell receptor；TCR)をもつT細胞レパートリーがもともと存在しないか，あるいは非常に少ない場合は，ペプチド特異的CTLの誘導が期待できず，細胞傷害性を示すことはできない．したがって，投与するエピトープが複数であれば，特異的CTLが誘導できる可能性がより高まる．②標的細胞の抗原提示の側面からみると，がん細胞はさまざまな抗原を提示しているが，一つの標的病変だけでみても，そのheterogeneityにより提示されるがん抗原エピトープも細胞個々で当然異なることが予想される．仮に3種のがん抗原由来ペプチドを投与して，それぞれに対する特異的CTLが誘導できたとすると，標的細胞が3種すべての抗原を提示していれば，複数の種類のCTLが認識し傷害できる可能性が考えられ，細胞傷害活性が高まると考えられる．標的細胞が1種の抗原のみ提示している場合でも，少なくともそのペプチド特異的CTLによる傷害が期待できる．単一のペプチドワクチンではこのような利点は期待できず，標的細胞に抗原提示されていなければ特異的CTLによる腫瘍拒絶には至らない(図3)．複数のエピトープでCTLを誘導することは，がん細胞の免疫逃避機構からの克服の観点からも理にかなっているものと思われる．

このように複数抗原ペプチドをカクテル製剤とすることで投与ペプチドの選択肢が広がり，抗腫瘍効果増強の期待も高まるが，一方で，カクテル製剤にすることによる負の側面も考えられる．まず，各ペプチドを溶解するための適切な溶媒の選択が必要となる．さらに，ペプチドを混合することで，ペプチド間の相互作用等によりペプチドに変性をきたさないか等の品質保

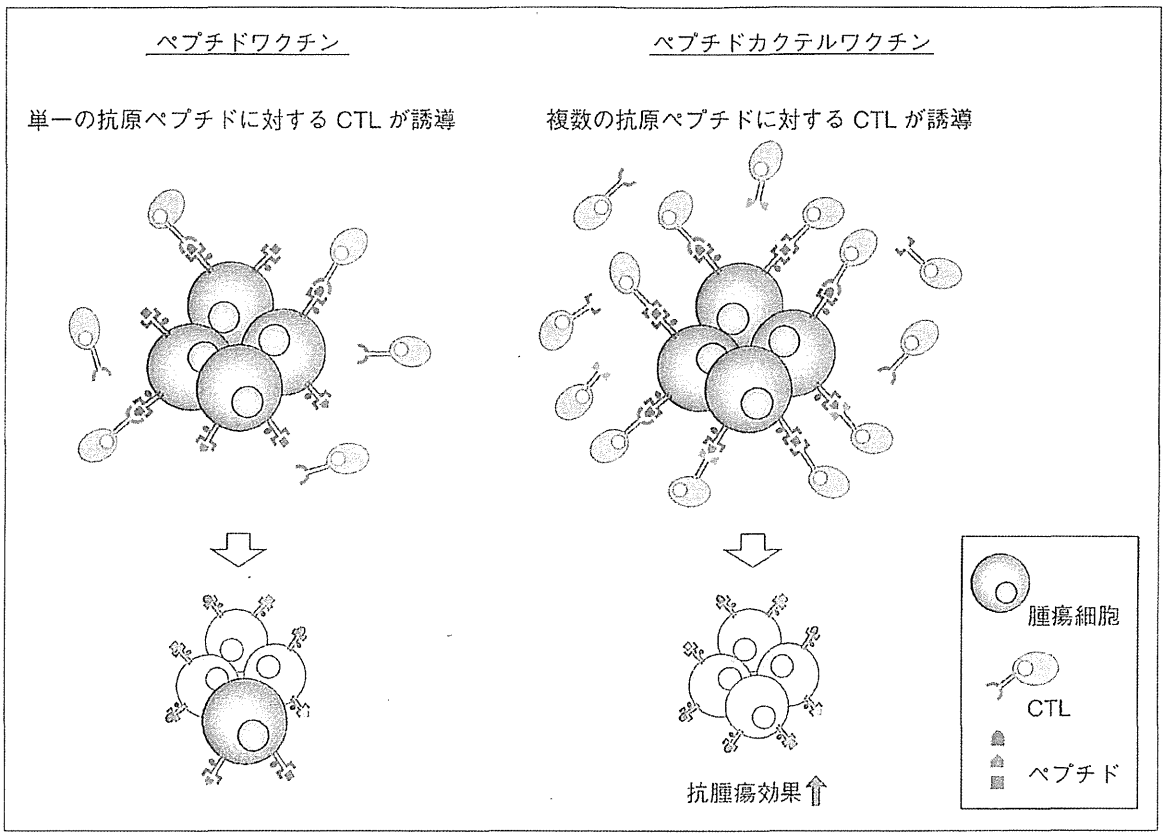

図3　単一抗原に対するペプチドワクチン療法とカクテルワクチン療法
単一抗原に対するワクチンでは単一のCTLが誘導される．カクテルワクチンとして複数のペプチドを投与することで複数抗原に対するCTLの誘導が期待される．標的細胞側には種々のペプチドが提示されているため，CTLが複数種類誘導されれば，CTLにより傷害できる可能性が高まる．

表1　がんペプチドカクテルワクチン療法における利点と不利点

利点	不利点
複数抗原に対するCTLを誘導可能	APC上のHLA－ペプチド間のaffinityに差が存在
CTLの誘導率の上昇	品質保持の確認が煩雑
標的細胞に対する傷害活性の増強	副作用増大の可能性
投与ペプチドの選択肢増加	ペプチドごとのDTH反応の評価が不可

持の確認がなされなくてはならず，ペプチド数が増えるごとに安定性の評価がより煩雑になる．次に，それぞれのMHC－ペプチド間のaffinityに差が存在するため，1か所に複数抗原に対するペプチドを投与した場合，各ペプチド間で抗原提示細胞上のMHC class I 分子との結合に差が生じることが予想され，CTL誘導に影響する可能性が考えられる．がんペプチドカクテルワクチン療法においては，affinityの差による特異的CTLの誘導効率については十分考慮されなくてはならない．複数抗原ペプチドをカクテル製剤として投与する際の利点および不利点を表に示す(表1)．

がんペプチドカクテルワクチン療法は，多彩な組み合わせが可能であり，CTLエピトープのみを用いたカクテル製剤[6)7)]や，CTLエピトープおよびヘルパーエピトープを含むカクテル製剤[5)8)]など，さまざまな臨床試験，開発がすすめられており，今後のがんペプチドワクチン療法開発の主流になるであろうことが予想される．標準治療不応の進行・再発食道がんに対する，TTK, LY6K, IMP-3の3種のペプチドワクチンを用いた臨床第II相試験では，高い忍容性が確認され，

HLAがマッチした群においてマッチしない群に比べ，統計学的な有意差には至らないものの全生存期間(OS)の延長がみられる傾向にあり〔生存期間中央値(median survival time；MST) 4.6か月 vs. 2.6か月〕，無増悪生存期間では，有意差をもって延長がみられた．さらには免疫学的解析において，1個<2個<3個と複数種類のペプチド特異的CTL反応が多く得られた群で，よりOSの延長がみられる傾向にあった[9]．また，標準療法抵抗性の再発大腸がんに対するtegafur-uracil/leucovorin(UFT/LV)との併用で行われたRNF43, TOMM34由来ペプチドを用いたペプチドカクテルワクチン療法臨床試験においては，RNF43, TOMM34両者に対するCTL反応が得られた群において，CTL反応がどちらか一方のみであった群に対して，有意にOSの延長がみられた(MST 34.2か月 vs. 12.9か月)[10]．また，欧米において10種のペプチドカクテルワクチン(IMA901)の第III相臨床試験が進行中である．この10種の抗原ペプチドはヒト腎細胞がん組織細胞膜表面への提示が確認されたエピトープペプチドであり，IMA901は9つのCTLエピトープと1つのヘルパーエピトープから構成される．腎細胞がんに対するIMA901の第II相臨床試験では，2種あるいはそれ以上のペプチドに対する免疫反応が得られたグループで，生存期間の延長が観察された[11]．これらの臨床試験の結果は，がんペプチドカクテルワクチン療法がより有望な選択肢となることを示すものであり，複数抗原ペプチドをカクテル製剤として用いることの一つの根拠となるものと思われる．

GPC3由来がんペプチドワクチン療法の取り組み

筆者らは，新規がん抗原としてGPC3を同定した[12]．GPC3は肝細胞がん，卵巣明細胞腺がん，肺扁平上皮がん等に発現し，小児がんにおいては，肝芽腫，腎芽腫，卵黄嚢腫等に発現がみられ，正常組織においては胎生期の肝臓，胎盤のみに高発現している．当院では進行肝細胞がんに対するGPC3由来ペプチドワクチンを用いた第I相臨床試験を行い，安全性を報告し，免疫学的解析において末梢血単核球5×10^5個のうち50個以上のペプチド特異的CTLが誘導できた群では，50個未満の群に比べOS中央値の有意な延長(12.2か月 vs. 8.5か月，$P=0.033$)がみられたことを報告した[13]．さらに，ワクチンによりCTLの腫瘍への浸潤が増加することを生検検体の解析で示した．このことは，ペプチドワクチン療法のproof of conceptを示した結果といえる．肝がんに対するGPC3ペプチドワクチン療法は臨床試験の結果をうけ企業への導出がなされ，GPC3ペプチドを含むカクテルワクチンの企業治験がスタートしている．また，再発予防効果の検証を目的とした，肝細胞がん根治的治療後の第II相試験が進行中であり，登録が完了している．他がん種では，卵巣明細胞腺がんに対する第II相試験が進行中であり，肝細胞がんと同様部分寛解(partial response；PR)症例もみられている．小児がんに対しても，GPC3を発現する肝芽腫等を対象とした多施設第I相試験が進行中であり，小児患者に対する安全性や至適用量決定の評価が待たれる．

難治性小児固形がんに対するがんペプチドカクテルワクチン多施設第I相臨床試験の概要

国立がん研究センター東病院では，当院を中心に多施設で難治性小児固形がんに対するKOC1, FOXM1, KIF20A由来ペプチドカクテルワクチンを用いた第I相の医師主導治験を2013年3月よりスタートさせた．対象年齢は1～40歳で，標準治療不応の進行，再発神経芽腫，Ewing肉腫ファミリー腫瘍，横紋筋肉腫，骨肉腫を対象疾患とした．これら対象疾患の治療法は外科的切除，化学療法，放射線療法などの集学的治療となる．治療成績は集学的治療により著明に改善されてきており，現在，小児がん全体では70%が治癒可能となった．しかし，約30%は難治性であり，再発した場合には5年生存割合が20～30%と予後不良である．再発後の治療として確立した治療法はなく，一般臨床では既存の薬剤を用いた治療(二次化学療法)が行われるが，その後の治療は試験的治療となる．このような難治性の小児腫瘍は100～200人程度/年であり，限られた数ではあるが，未来を担う小児を一人でも多く救

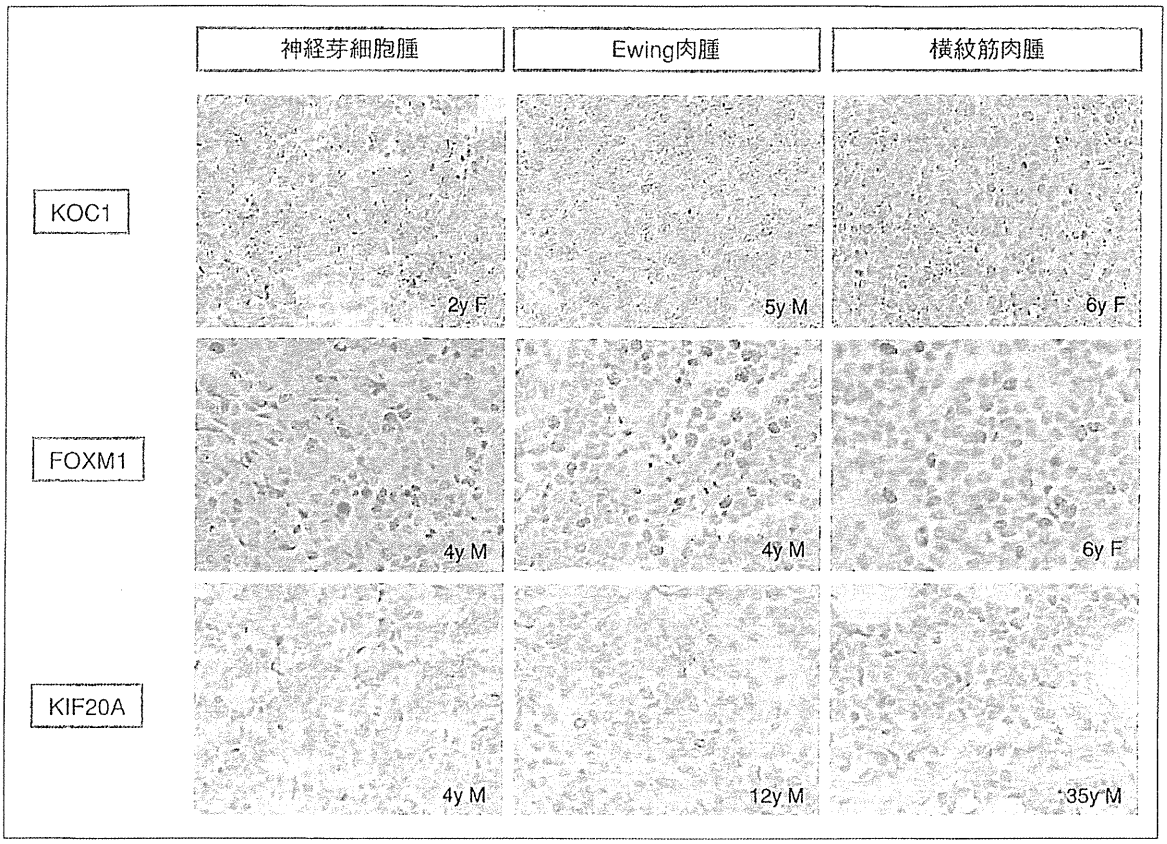

図4　各がん種と抗原の発現(免疫染色)

済できる治療法を開発することは，重要な課題であると筆者らは考えている．

難治性小児固形腫瘍の治療開発としては，新規薬剤開発，放射線治療の改良などが多く試みられているが，開発コストの問題や期待される有効性に達しない等の理由から後期開発に進む治療はほとんどない．一方，免疫療法は臨床研究としては20年以上前から検討が続けられてきた．最近では，神経芽腫に対して，標準療法に加え，抗GD2抗体とIL2/GM-CSFの併用療法が予後を延長したことが報告されている[14]．骨肉腫に対しては，単球，マクロファージを活性化するmifamurtide(MEPACT®)の初発時標準治療との併用下でのOS，無増悪生存期間の延長効果が示され[15]，2009年に欧州医薬品庁において非転移性骨肉腫に対し認可されるなど，小児難治性悪性腫瘍に対する治療開発戦略において，がん免疫療法は大きな比重を占めるに至っている．また，ペプチドワクチン療法では，本邦において，小児横紋筋肉腫に対するWT1ペプチドでの寛解例の報告もみられている[16]．

本試験において用いるNCCV Cocktail-1はHLA-A24拘束性KOC1，FOXM1，KIF20A由来ペプチドのカクテル製剤である．小児固形腫瘍におけるKOC1，FOXM1，KIF20Aの発現について報告はないが，自験例において，ほとんどの小児固形腫瘍で高い発現率を呈していた．具体的には，免疫組織染色においてKOC1，FOXM1については，神経芽腫5名中5名，Ewing肉腫ファミリー腫瘍5名中5名，横紋筋肉腫6名中6名，骨肉腫5名中5名，KIF20Aについては，神経芽腫5名中4名，Ewing肉腫ファミリー腫瘍5名中5名，横紋筋肉腫6名中6名，骨肉腫5名中5名で発現を確認した(図4)．以上の結果をふまえ，小児固形腫瘍患者の中でも，ほとんどの神経芽腫，Ewing肉腫ファミリー腫瘍，横紋筋肉腫，骨肉腫にKOC1，FOXM1，KIF20A発現が確認されることから，これらの疾患に対して上記3種類のがん抗原由来のがんペプチドカクテルワクチン療法の開発が可能と判断した．カクテルワク

チン療法とした理由は上述した通りである．本試験が成功し，企業治験としての第II相試験に引き継がれ，難治性小児がん患者の恩恵となればこの上ない喜びである．

おわりに

FDAは，2009年9月にがん治療用ワクチンに対する臨床的考察としての企業向けガイダンスードラフト版を発表し，2011年10月に最終版を発表した[17]．最終版にはmulti-antigen vaccinesの項が新たに設けられ，以下のごとく述べられている．「複数の腫瘍特異的な免疫応答を惹起し，潜在的な腫瘍逃避機構を回避するため，がんワクチン製剤は，複数の腫瘍関連抗原を含む場合がある．一般的に，複数の腫瘍関連抗原を含むがんワクチン製剤の各成分に関しては，個別に安全性と活性を評価する必要はない．ケースバイケースである．」このように複数抗原ペプチドワクチンの投与については，FDAからも言及があり，新規ワクチン療法としての開発がさらに期待される．現在本邦においては，がん治療に対し認可されたワクチン療法はなく，実用化にはランダム化比較試験において，その有効性を実証しなければならない．がん免疫療法は現在精力的に開発がすすめられており，がんペプチドカクテルワクチン療法が，今後のがん治療の一端を担うことを期待したい．

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Novel and rapid enumeration method of peripheral blood stem cells using automated hematology analyzer

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Keywords
PBSCT, HPC, automated hematology analyzer, hematopoietic stem cell transplantation

SUMMARY

Introduction: The number of infused $CD34^+$ cells is crucial to the success of peripheral blood stem cell transplantation (PBSCT). Here, we present, for the first time, a new method of enumerating hematopoietic progenitor cells (HPCs) for PBSCT.
Method: This novel method is based on hemolysis and chemical staining, followed by flow cytometry-based optical detection, conducted using an automated hematology analyzer (XN series, Sysmex). $CD34^+$ cells and HPCs were compared in 76 granulocyte colony-stimulating factor (G-CSF)-mobilized blood or apheresis samples taken from healthy donors (n = 18) or patients undergoing autologous PBSCT (n = 6).
Results: There was a strong correlation between the numbers of HPCs and $CD34^+$ cells (R^2 = 0.958). The expected total number of HPCs in the final products, which was estimated from HPCs in pre-apheresis PB or mid-apheresis products, also correlated well with the total number of $CD34^+$ cells in the final products. The change in HPCs in PB closely resembled that of $CD34^+$ cells during mobilization. Experiments using immunomagnetic beads suggested that the majority of $CD34^+$ cells existed in HPCs, and vice versa.
Conclusion: Hematopoietic progenitor cells may serve as surrogates for $CD34^+$ cells in PBSCT. However, further investigations are required to verify this.

INTRODUCTION

The number of infused $CD34^+$ cells is crucial to the success of peripheral blood stem cell transplantation (PBSCT) [1–3]. Although the ability to count $CD34^+$ cells currently depends solely on flow cytometric analysis, the complexity of the procedure and cost of reagents, including monoclonal antibodies, are major disadvantages. Biochemical examination using the hemolysis reaction and staining with a specific dye is another tool that has been used for many years as a mainstay for cell classification; most automated hematology analyzers utilize specific biochemical properties for counting and classifying blood cells [4, 5]. As blood cells mature and differentiate, the constituents of the cell membrane, cytoplasm, and intracellular granules change, and hemolysis-resistant properties depend on the degree of maturation. This is the basic idea behind the identification of immature cells with an automated hematology analyzer without using monoclonal antibodies [6].

Recently, this technology has been applied in practical use as the hematopoietic progenitor cell (HPC) counting method on the Sysmex SE-9000 (SE) and XE-2100 (XE) automated hematology analyzers. The SE/XE analyzers quickly estimate the number of immature cells, referred to as HPCs, at a very low cost [6, 7]. The number of SE/XE-determined HPCs in the peripheral blood (PB) is used to determine the optimal timing of PBSC collection [8–22]. The SE/XE-determined number of HPCs has also been shown to correlate well with the $CD34^+$ cell yield in apheresis products [8, 9, 13, 16–18]. However, the number of SE/XE-determined HPCs is limited as a substitute for the $CD34^+$ cell count [20, 21] because it is likely to be affected by coexisting immature cells (e.g., immature granulocytes), resulting in under- or overestimation of the number of HPCs in some situations [12–14, 22].

The newly launched XN-series model automated hematology analyzer (Sysmex Corporation, Kobe, Japan) is equipped with a white precursor cell (WPC) channel to detect immature cells, such as myeloblasts and abnormal lymphocytes. The measuring principle of this channel utilizes the optical detection system and general flow cytometry. In this channel, two reagents are used, that is, the lysis reagent (Lysercell WPC, Sysmex) containing a polyoxyethylene nonionic surfactant and the stain reagent (Fluorocell WPC, Sysmex) containing a polymethine dye. We optimized the hemolysis conditions, including reaction time, temperature, to identify PBSCs with reference to $CD34^+$ cells. Finally, we developed a new technology for counting HPCs based on hemolysis reactions with the surfactant and chemical staining with a specific dye.

The purpose of this study was to evaluate the XN-determined HPCs in reference to $CD34^+$ cells. This is the first report to investigate this topic.

MATERIALS AND METHODS

Study design

Healthy PBSCT donors and patients with hematological malignancies undergoing autologous PBSCT at the National Cancer Center Hospital, Japan, were enrolled into this study. PBSC and PB samples were taken from each donor or patient during granulocyte colony-stimulating factor (G-CSF) mobilization or the bone marrow recovery phase after chemotherapy. HPCs and $CD34^+$ cells were examined in the same fresh samples and were subsequently compared. The primary objective was to examine whether there was a correlation between HPCs and $CD34^+$ cells in PB and PBSC samples. We also investigated whether it was possible to predict the total number of $CD34^+$ cells in the final product from HPCs in the PB immediately prior to the start of apheresis and from HPCs in the PBSC sample taken during an apheresis session. The kinetics of HPCs and $CD34^+$ cells were also examined by taking PB samples at several time points during PBSC mobilization to examine whether the correlation between these two markers was constant at all time points. The relationship between the cell populations defined by HPCs and $CD34^+$ cells was investigated using $CD34^+$ cell-enriched or cell-depleted PBSC samples, which were obtained by isolation with immunomagnetic beads. This study was approved by the institutional review board, and informed consent was obtained from each patient or donor.

PBSC mobilization and apheresis

Healthy donors were administered G-CSF subcutaneously at a dose of 10 μg/kg per day, and apheresis was started on the fourth or fifth day until the target total

number of 2×10^6 $CD34^+$ cells per kg of patient body weight was obtained. Patients underwent apheresis until the same target number of $CD34^+$ cells was obtained during the bone marrow recovery phase after chemotherapy under the support of intravenous G-CSF (5 μg/kg/day). Apheresis was performed using the COBE Spectra cell separator (Terumo BCT, Lakewood, CO, USA) with 10 L of blood processed in each subject. An acid–citrate–dextrose A (ACD-A) solution (Terumo, Japan) mixed with heparin sodium at a concentration of 5 U/mL was used for anticoagulation during apheresis.

Measurements of HPCs

Hematopoietic progenitor cells were measured using the Prototype XN-series automated hematology analyzer (Sysmex) with the WPC channel. Additional software for HPC counting was installed on the instrument without modifying the hardware or reagents of the released product model. The lysis reagent for this channel contained a surfactant. Lipid components, constitutive elements of the cell membrane, were extracted by the surfactant, and minute pores were formed, resulting in reduced stability of the cellular structure and shrunk or broken cells [23–25]. Then, dyes migrated into the cells through the openings formed by the surfactant and bound to nucleic acids or cytoplasmic organelles. The greater the damage to the leukocytes, the more easily they were stained. The lipid content of the membranes of white blood cells (WBCs) has been shown to increase with maturation, and mature neutrophils contain the highest levels of phospholipids [26–28]. Therefore, mature neutrophils were more likely to be damaged than immature leukocytes by incubation with reagents containing a surfactant. The rising fluorescence intensity of cells after incubation was associated with the degree of cell membrane damage. Thus, these biochemical reactions highlighted cellular characteristics associated with the membrane, cytoplasm, and intracellular granules of each cell type on the basis of their unique staining patterns and morphological features. Finally, three signals were produced by the optical detection system: forward scattered light, providing information on cell size; side scattered light, providing information on internal cell structure; and side fluorescence, providing information on the degree of dye staining. The results were shown as the HPC concentration (cells/μL) on the screen with a scattergram of the HPC analysis. HPCs were defined as dots that showed higher light intensity than lymphocytes in the forward scatter, which reflected the cell size, and equal light intensity as lymphocytes in the side scatter, which reflected the complexity caused by the presence of intracellular granules. In addition, they were also defined as dots that showed lower light intensity than lymphocytes in the side fluorescence scatter, which reflected the degrees of resistance to the surfactant and maturation (Figure 1). In the WPC channel, 4 μL of sample was used for HPC analysis, and the whole assay required <200 μL volume, <5 min time, and no monoclonal antibodies.

Measurements of $CD34^+$ cells

$CD34^+$ cells were quantitated using the dual platform method according to the protocol of the International Society of Hematotherapy and Graft Engineering (ISHAGE) [29, 30]. Briefly, cells were double-labeled with phycoerythrin (PE)-conjugated anti-CD34 and fluorescein isothiocyanate (FITC)-conjugated anti-CD45 monoclonal antibodies (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using a flow cytometer (FACSCalibur, Becton Dickinson). The number of $CD34^+$ cells was calculated from the percentage of $CD34^+$ cells with reference to $CD45^+$ and WBC counts. The WBC count was obtained using the XE-2100 automated hematology analyzer (Sysmex). The concordance of $CD34^+$ cell counts between dual- and single-platform methods was confirmed using fresh samples obtained from mobilized blood or apheresed products in our laboratory.

Enrichment of $CD34^+$ cells by using immunomagnetic beads

$CD34^+$ cells were isolated using MACS magnetic cell separation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Two independent isolation experiments were performed by using different methods, that is, direct isolation of CD34-expressing cells (positive selection) and depletion of lineage-positive cells (negative selection). Positive selection was performed using the Indirect CD34 MicroBead Kit (Order no. 130-046-701; Miltenyi Biotec, Bergisch Gladbach, Germany),

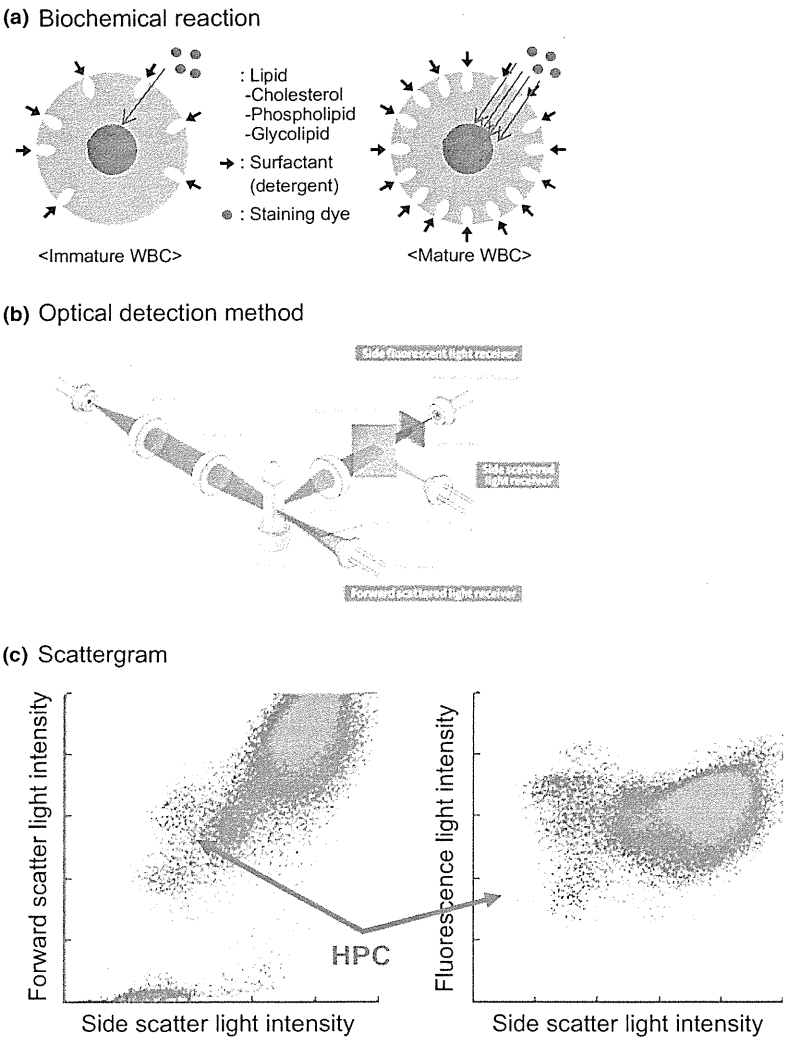

Figure 1. Principle of the HPC enumeration method. (a) The biochemical reaction, including hemolysis and staining with fluorescent dyes, discriminated immature leukocytes because of the differences in the resistance to the surfactant, which was dependent on the lipid contents within the cell membrane. (b) An optical detection method based on flow cytometric analysis was used. (c) The scattergram shows leukocytes as light blue dots and the HPC fraction as purple dots. HPCs were defined by a fixed gate, which was determined from three parameters.

according to the manufacturer's instructions. Briefly, cells were incubated with CD34–hapten–antibodies and an FcR-blocking reagent. After washing with MACS buffer, the cells were incubated with antihapten-conjugated MicroBeads followed by incubation with PE-conjugated anti-CD34 antibodies. After washing, cells were applied onto a column attached to a magnet. The labeled cells, which were trapped in the column within the magnetic field, were retrieved by washing the column after it was removed from the magnet. A Lineage Cell Depletion Kit (Order no. 130-092-211; Miltenyi Biotec, Bergisch Gladbach, Germany) was used for negative selection. This kit consisted of a biotin-conjugated antibody cocktail (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a) and antibiotin-conjugated MicroBeads. Labeling and column separation were performed in the same way as in positive selection. Enriched $CD34^+$ cells were collected as unlabeled cells.

Statistics

Statistical analysis was performed using SPSS 15.0J software (SPSS Inc., Chicago, IL, USA). Correlations were assessed with Pearson's correlation test. Differences between the two groups were assessed by using the nonparametric Wilcoxon signed rank test. *P*-values of <0.05 were considered statistically significant. Differences in the slope of linear regression equations between the two groups were assessed by parallel tests.

RESULTS

Patients and materials

Between 2008 and 2011, a total of 18 healthy donors and 6 patients were enrolled into this study. Seventy-six mobilized blood and apheresis products were used for the analysis. The diagnosis was non-Hodgkin's lymphoma in all patients.

Basal performance of HPC enumeration

The feasibility of the basal performance of HPC enumeration, including reproducibility, sample stability, linearity, and the effects of anticoagulant, was evaluated and confirmed using mobilized blood and apheresis products.

The reproducibility of HPC counts was evaluated by five repeated analyses using five different PB samples (HPC range, 22–59 cells/μL), and the coefficient of variation (CV) was 10.3% (range, 5.6–16.1%). Concerning the sample stability, HPCs were evaluated periodically using independent PB samples at room temperature over 6 h after sampling, and stability was maintained. We also compared HPC counts in PB samples that had been stored for over 24 h with those in fresh samples, and there were no significant differences (data not shown). This suggested that cell viability did not influence HPC counts significantly, at least for 24 h from sampling. The linearity, which was evaluated using diluted PB or apheresis products, was almost concordant with the theoretical values (R^2 = 0.996, slope of the regression curve: 0.981). There were no significant differences between apheresis samples with or without EDTA-2K as an anticoagulant.

Correlation between HPCs and CD34$^+$ cells

CD34$^+$ cell counts ranged widely. The median and range in the pre-apheresis PB and intermediate and final apheresis products were 25.9 cells/μL (range, 0.3–453.5 cells/μL), 955.0 cells/μL (range, 379.4–12 830.5 cells/μL), and 597.3 cells/μL (range, 67.1–11 805.3 cells/μL), respectively. There was a very strong correlation between the numbers of HPCs and CD34$^+$ cells in all samples (R^2 = 0.958). Strong correlations between these two markers were also observed in both subgroups of the pre-apheresis PB (R^2 = 0.919) and apheresis products (R^2 = 0.958; Figure 2). There were no statistically significant differences between the slopes of regression equations in PB and apheresis products (P = 0.729).

Next, we examined the possibility of predicting the total number of collected CD34$^+$ cells from pre-apheresis PB. From HPCs in pre-apheresis PB, the expected HPC yield was calculated as follows: expected HPC yield = [HPCs in PB (cells/μL)] × [processed blood volume (mL)] × 10^3 × [collection efficiency], where the processed blood volume was 10 L in all apheresis sessions. The expected HPC yield correlated with the number of CD34$^+$ cells in the final products (R^2 = 0.952), if the collection efficiency was assumed to be constant (50%). The HPC count in mid-apheresis PBSC products also had a good correlation with the CD34$^+$ cell count in the final product (R^2 = 0.918; Figure 3). The slopes of both regression equations on expected HPCs were not significantly different (P = 0.156).

Concerning the kinetics of HPCs and CD34$^+$ cells in the PB during mobilization, the changes in HPCs were similar to those in CD34$^+$ cells in all nine patients and donors (Figure 4). The timing of appearance of HPCs in the PB was almost concordant with that of CD34$^+$ cells, which suggested that the appearance of HPCs may be a good indicator for the optimal timing of PBSC collection.

Relationship between HPCs and isolated CD34$^+$ cells

Apheresis products were processed immediately after sampling. To clarify the relationship between HPCs and CD34$^+$ cells, CD34$^+$ cells were isolated from the final products by positive or negative selection using immunomagnetic beads. First, because the attachment of immunomagnetic beads may affect the results of HPC counts, CD34$^+$ cells were enriched by depleting lineage-positive cells. The resulting lineage-negative cells showed two major populations in the HPC scattergram: one population (20.6%) in the HPC area and another in the area corresponding to granulocytes in a representative experiment (Figure 5a). Flow cytometric analysis also showed two populations in the forward *vs.* side scattergram; one population, which accounted for 22.8%, corresponded to CD34$^+$ cells, and the other population comprised cells that were

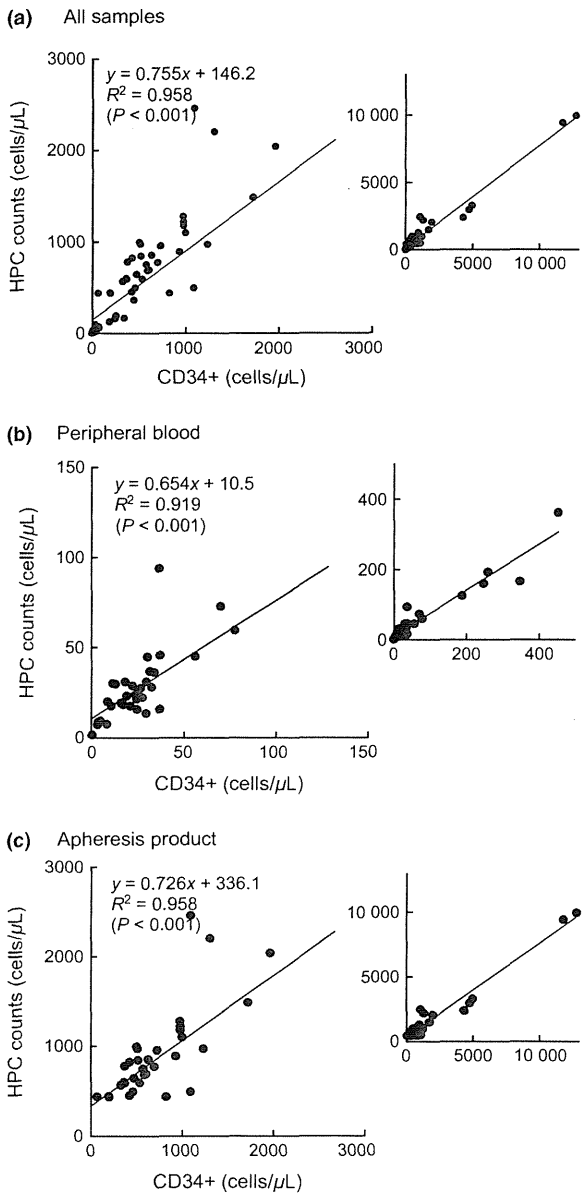

Figure 2. Correlation between HPC counts and the number of CD34$^+$ cells. (a) Samples included the PB and apheresis products (n = 76). Values below 3000 cells/μL are plotted separately (lower left), in addition to all PB samples (upper right). (b) PB samples. Values below 150 cells/μL are plotted separately (lower left), in addition to all PB samples (upper right). (c) Apheresis products. Values below 3000 cells/μL are plotted separately (lower left), in addition to all apheresis samples (upper right).

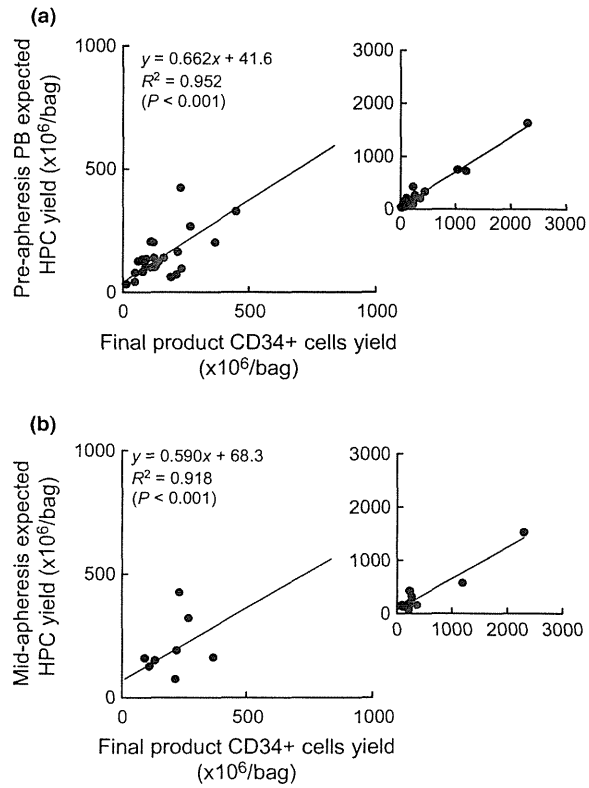

Figure 3. Correlation between expected HPC yield and true CD34$^+$ cell yield. The expected HPC yield was calculated or estimated from HPCs in PB (n = 28) (a) or mid-apheresis products (n = 10) (b). Values below 1000 × 10^6 cells/bag are plotted separately (lower left), in addition to all samples (upper right).

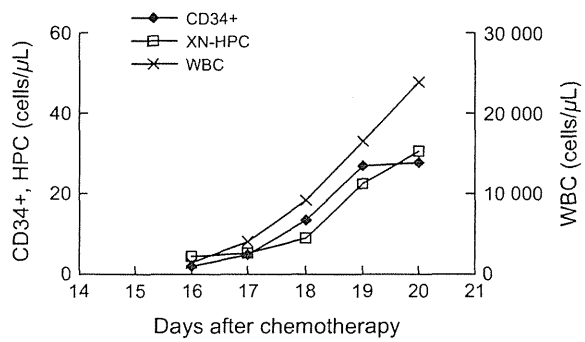

Figure 4. Changes in CD34$^+$ cells, XN-determined HPCs, and WBCs in the PB during the recovery phase from myelosuppression after chemotherapy in a representative patient.

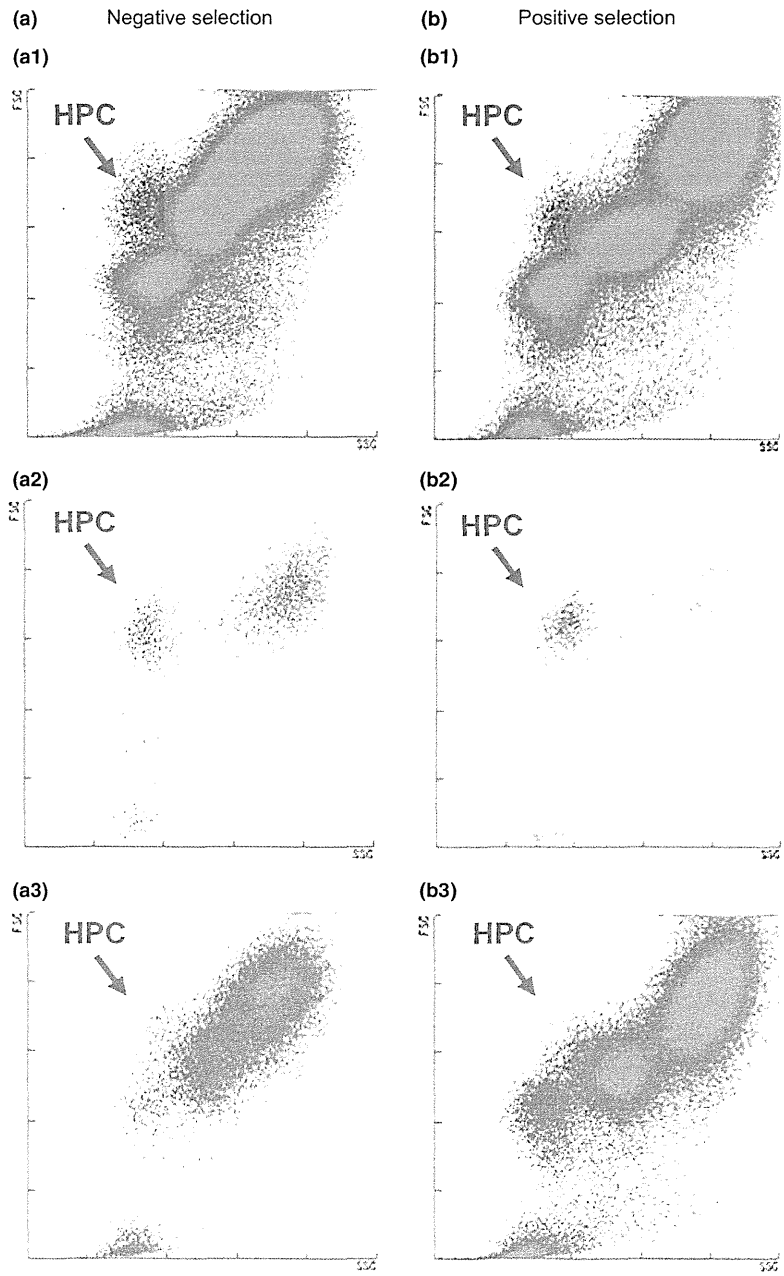

Figure 5. HPC scattergram of immunomagnetically enriched or depleted $CD34^+$ cells in representative experiments. (a) $CD34^+$ cells were enriched from apheresis products by depleting lineage-positive cells (negative selection). (a1) Before separation: $CD34^+$ 0.6%, HPCs 0.7%. (a2) Lineage-negative fraction: $CD34^+$ 22.8%, HPCs 20.6%. (a3) Lineage-positive fraction: $CD34^+$ 0.4%, HPCs 0.5%. (b) $CD34^+$ cells were isolated directly using anti-CD34 antibodies. The location of dots indicated that HPCs may have shifted from their original position. (b1) Before separation: $CD34^+$ 0.5%, HPCs 0.4%. (b2) $CD34^+$ fraction: $CD34^+$ 83.2%, HPCs 70.2%. (b3) CD34-depleted fraction: $CD34^+$ 0.1%, HPCs 0.2%. Dots detected in the HPC area appeared to correspond to $CD34^+$ cell populations, independent of the separation method.

negative for CD34 expression. Microscopic examination of this fraction after cytospin preparation followed by May–Giemsa staining revealed that there were also two major cell populations; these cell populations were mainly composed of promyelocytes (>50%) and blastoid mononuclear cells (approximately 40%). On the other hand, lineage-positive cells, including few $CD34^+$ cells (0.4%), showed few dots in the HPC area (0.5%).

Next, $CD34^+$ cells were directly isolated with anti-CD34 antibodies and analyzed for HPCs. The positively isolated fraction with a $CD34^+$ cell purity of 83.2% showed a single cluster in the HPC area (70.2%) of the scattergram, while the CD34-depleted

fraction, including 0.1% $CD34^+$ cells, appeared as very few dots in this area (HPCs, 0.2%; Figure 5b).

DISCUSSION

Automated hematology analyzers are usually used in routine laboratory tests. Several authors have reported that the Sysmex SE/XE-series HPC count system is a useful additional function of the automated hematology analyzer [8–22]. However, it has the disadvantage of low counting performance, which is mainly caused by the lack of ability to identify cell populations precisely. In the XN-series model, the performance has been improved by changing the reagent composition and the detection principle. The cell population corresponding to HPCs in the XN-series model is identified by a flow cytometry-based optical detection system that differs from the previous SE- or XE-series hematology analyzers, which utilize the electrical radiofrequency (RF)/direct currency (DC) impedance detection method. The optical detection system allows us to more accurately recognize and differentiate the morphological and biochemical properties of each cell based on the degree of staining. Moreover, the volume of sample required for HPC analysis has been increased from 1 to 4 μL per assay, which has increased the accuracy of HPC, especially at lower concentrations.

In this study, we demonstrated that there was a good correlation between the numbers of HPCs and $CD34^+$ cells in samples from not only the PB but also apheresis products. The correlation coefficient was much closer to 1.0 with this novel XN-determined HPC count than with the previous SE/XE-determined HPC count [8, 9, 13, 16–18, 20, 21]. These results suggested that the number of $CD34^+$ cells in the final products could be predicted from the HPC count in the final products. The correlation was unaffected by the WBC counts, use of EDTA as an anticoagulant, sample types (i.e., PB or PBSC), or timing of collection. Moreover, our data from the experiments using immunomagnetic beads indicated that HPCs reflected the majority of cells in the $CD34^+$ cell fraction. As for the markers of the clinically relevant number of hematopoietic stem cells required for a successful PBSCT, only the numbers of colony-forming cells [31] and $CD34^+$ cells [3, 32–34] have been used so far [2, 35]. Our data suggested the possibility that the number of HPCs may be another candidate marker of hematopoietic stem cells in PBSCT. Secondly, the approximate number of $CD34^+$ cells in the final products may be predicted from the HPC count in pre-apheresis PB. Moreover, the fact that the change in the number of HPCs closely resembled that of $CD34^+$ cells in the PB during mobilization also suggested that HPCs in the PB may be a good indicator for the optimal timing of PBSC collection, as is $CD34^+$ cell concentration [32, 33, 36] and SE/XE-determined HPC counts [9–22]. Thirdly, our data also suggested that the final $CD34^+$ cell yield may be predicted from the HPC count in the mid-apheresis product. This may allow us to adjust the planned total processed blood volume during apheresis so that an optimal and sufficient apheresis session can be accomplished.

Concerning the cell population corresponding to HPCs, we demonstrated in our experiments using immunomagnetic beads that the majority of HPCs overlapped with the $CD34^+$ cell population in the PB or PBSCs. HPCs cannot be retrieved intact because the cell membrane will be damaged or lysed during the assay process. Moreover, any manipulation of cells, whether immunomagnetic beads are used or not, will not allow us to accurately enumerate HPCs because the hemolysis reaction is very sensitive and because the location of the HPC fraction in the scattergram may shift. Because the HPC population was determined by fixed gating in the HPC scattergram of the XN-series automated hematology analyzer, even a subtle shift in the scattergram may result in false HPC values. Therefore, we first evaluated HPCs by using PBSC samples in which $CD34^+$ cells had been enriched by depleting lineage-positive cells without direct attachment to immunomagnetic beads and revealed that the concentrations of $CD34^+$ cells and HPCs were almost equal. Next, the fact that $CD34^+$ cells, which had been isolated using anti-CD34 antibody-conjugated immunomagnetic beads, showed a single cell fraction around the HPC gate in the scattergram, suggesting that most $CD34^+$ cells in this sample fell into the HPC gate. The percentages of HPCs and $CD34^+$ cells may reflect a shift after processing of the cells, including direct attachment to immunomagnetic beads, centrifugation, cell wash. These results demonstrated that the majorities of HPCs and $CD34^+$ cells were almost identical, at least if the sample was derived from mobilized PB or apheresed PBSCs.

Theoretically, in the very primitive stem cell fraction, the number of $CD34^-$ stem cells, whether HPCs are included or excluded, is considered very small and negligible [37–39]. In the fraction of more mature myeloid cells, it is reasonable to speculate that the majority of HPCs exist in the less-differentiated fraction than in the most-differentiated CD34-weakly expressing progenitor cells, which are infrequently observed in the mobilized blood.

Because this method has some advantages, such as rapid assay time, ease, and low cost, it is possible that enumeration of $CD34^+$ cells may be substituted for that of HPCs for determining an optimal stem cell dose for PBSCT. Before the era of $CD34^+$ cell enumeration, the number of granulocyte–macrophage colony-forming cells (GM-CFCs) was the main marker for PBSCT [29]. Although there is a good correlation between the numbers of GM-CFCs and $CD34^+$ cells, there is an almost 10-fold difference in quantity; in contrast, the number of HPCs is more similar to that of $CD34^+$ cells. Moreover, the use of fixed gating and the operability on an automated hematology analyzer will facilitate the standardization of HPC enumeration protocols.

In conclusion, we developed a novel HPC enumeration method, carried out without using monoclonal antibodies on an XN-series automated hematology analyzer. The final amount of collected $CD34^+$ cells may be predicted from the total number of HPCs in the final products, as well as from pre-apheresis PB and intermediate products during apheresis. HPCs may also be a good indicator for the optimal timing of PBSC collection. Because the number of patients in this study was small, interpretation of the results is limited. Therefore, we are currently conducting a multicenter prospective study, in which a larger number of patients and healthy donors will be enrolled, to determine the usefulness of HPCs in practice.

ACKNOWLEDGEMENTS

RT, TK, AY, and SO designed the research project, carried out the experiments, and analyzed the data. YH contributed to the data analysis and organization of the study. AN, NT, and SY helped with sampling. RT, AY, and SO wrote the manuscript. All the authors interpreted data, reviewed the manuscript, and approved the final version. This study was conducted in collaboration with Sysmex Corporation, Kobe, Japan.

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