が大きいもの，③パニックや社会の混乱を引き起こすもの，④公衆衛生上特別の準備を要するものであるとし，これらをカテゴリーA（表2）に分類している。最も脅威となる生物剤は炭疽菌と天然痘ウィルスである。肺炭疽と天然痘の死亡率は85%と35%と高い。肺炭疽は潜伏期が1－5日と短く，発病後は抗生物質を投与しても1－3日で死亡する。天然痘はペストと並び伝染性が強い。ペストと野兎病（ツラレミア）は致死率は高いが，抗生物質を用いた有効な治療法が確立している。一方，フィロウィルス由来のウィルス性出血熱（エボラ出血熱，マールブルグ熱）は伝染性や致死率が共に高く，有効な予防手段や治療手段も確立していない。ボツリヌス毒素は，毒性は高いが，量産が難しく安定性を欠くため，影響を及ぼしうる地域が限られている[5]。

一般的に化学剤と比して生物剤は無臭で潜伏期があるため，生物剤攻撃は自然感染との区別が難しく秘匿的である。また犠牲者の分布は広がり，その伝染性により2次被害も大きく拡大する。表3に化学剤と生物剤の違いについて記述した[6]。

表2．CDCの示した対処準備の優先順位の高い生物剤（カテゴリーA）

Variola Major（天然痘）
Bacillus Anthracis（炭疽）
Yersinia Pestis（ペスト）
Clostridium Botulinum Toxin（ボツリヌス毒素）
Francisella Tularensis（ツラレミア菌）
Filoviruses（エボラ出血熱，マールブルク熱）
Arenaviruses（ラッサ熱，アルゼンチン出血熱）

文献6より引用。

生物化学剤の除染

1．化学剤除染

化学剤除染とは，問題となる部位・場所に化学剤が存在する場合に，何らかの方法で化学剤が人体に影響を及ぼさない程度，できれば存在しない状態にまで減少させることである。除染法として表4のように物理的除染と化学的除染があり，地域除染，装備品等の除染，個人除染，さらに曝露した皮膚及び創傷の除染がある[4]。

表3．化学剤と生物剤の違い

	化学剤	生物剤
潜伏期	速効性	潜伏期がある
性状	有臭～無臭	無臭
	揮発性～残留性	揮発性なし
露見性	露見的	露見的～秘匿的 自然感染との区別が重要
犠牲者の分布	散布場所に限局	潜伏期の間に分散
2次被害	患者に付着した残留物質に接触した場合	種類によりヒト→ヒト感染により拡大
除染の必要性	必要	状況により必要

文献6より引用，改変。

表4．物理的及び化学的除染法

除染方式	原理	具体的方法
物理的除染	機械的除去	拭き取る，拭い取る，汚染した衣類の除去
	液体による洗い流し・希釈	流水による洗浄，洗い流す，水に浸す
	粉体による吸着	除染用の吸着剤による吸着，小麦粉等の吸水性粉体による吸着
化学的除染	酸化	活性塩素 （次亜塩素酸ナトリウム水溶液，次亜塩素酸カルシウム水溶液，さらし粉水溶液等）
	加水分解	アルカリ性水溶液，石鹸水，水道水

文献4より引用，改変。

物理的除染に共通する利点は非特異的であることで，化学剤の構造に係らず，みな同等に効果を示す。他方，化学的除染には「酸化」と「加水分解」の2種類の化学反応が利用されている。マスタード（HD）やV剤（VX）は分子内に硫黄を有し，容易に酸化される。また，VX，タブン（GA），サリン（GB），ソマン（GD）等の神経剤は分子内にリンを有し，この部位で容易に加水分解される。このような理由で化学剤の除去剤は，酸化及び加水分解の両者の効果を持つようにデザインされている[1,4]。

アルカリ性にした次亜塩素酸塩水溶液は，現時点で酸化と加水分解性を併せ持つ優れた化学剤の除染剤として世界中で採用されている。米軍においては，0.5%の次亜塩素酸ナトリウム又は次亜塩素酸カルシウムを皮膚の除染，5%のものは装備品等の除染に使用されている。次亜塩素酸塩水溶液は，健常皮膚のみに使用可能で，外傷部や眼球部位，或いは腹腔内や胸腔内等内用に使用することはできない。さらに次亜塩素酸塩は毒性が強く，また溶解や洗浄に大量に水を必要とするため，本剤を大量に用いることは環境に与える影響やそのコストーパフォーマンスも考慮する必要がある。また洗い流した残存化学剤や大量の次亜塩素酸塩溶液の排水を既存の排水溝に垂れ流すことは許されず，洗浄排水処理の問題が発生する[1,4]。

化学剤の除染については，除染の効果のための評価法を構築する必要がある。除染が確実に行なわれたかどうかの確認は，いつに化学剤検

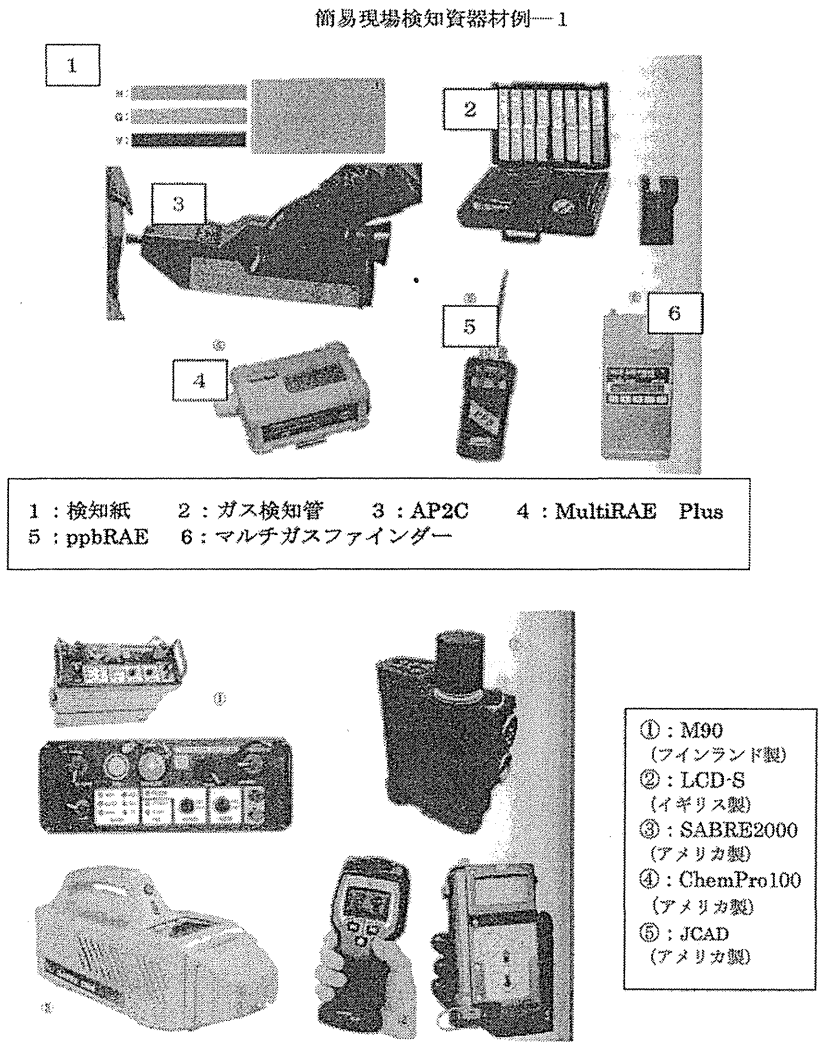

図1．簡易現場検知資器材（可搬・汎用性）
文献7より引用，改変。

知・分析法にある。部隊隊員の臭覚や小鳥（カナリア）等による有害化学物質の簡易検知や検知紙の適用から，現場検知器としては，初動対処部隊が所持し移動しながら検知する「小型携帯型」，現場で固定して検知する「可搬型」，設置場所に固定して連続モニタリングを目的とする「設置型」に区分される。化学剤検知のため現場で使用できる「小型携帯型」としての簡易現場検知資器材例[7]を下記に示す（図1）。さらに設置型や携帯型を組み合わせて，化学剤・毒素の一斉現場検知システムの開発が望まれる。

適切な手段で除染が実施されたなら，負傷者が汚染された状態で病院等へ搬入される可能性は極めて低くなる。また医療者や周囲の人間が危険に曝露する可能性も一層激減する。

2．生物剤の除染

生物剤の除染とは，個人の身体，衣類・装具，物品，床・壁等に付着ないし集積した汚染物質又は病原体を除去又は無害化することをいう。化学剤と異なり，生物剤は皮膚等に付着しても直ちには無害であり，除染の緊急性・必要性は低いと言える。生物剤対処における除染の主な目的は，防護衣や防護器材なしでも医療関係者が感染することなく安全に患者を取り扱えるようにすることである。秘匿的攻撃の場合は，テロ攻撃に気づいた頃には，すでに環境や体表から生物剤は消失し，除去の時期を失している可能性が高い。この場合は除染よりも病原体を同定し疫学的感染症対策による感染防止の施策が必要となる[6]。

最近の除染剤の動向

実際に運用されている除染方法に加えて，民間や米軍などで開発された除染剤，研究開発中で論文などに発表されている有益・有望技術について紹介する。

（1）ハイパー・イオン水　（ワールド・エコロジー（株））

深海からとった深層水を処理した粉末で，水で溶解したアニオンではPh 13.6以上，カチオンでは1以下であり，強い殺菌作用及び加水分解作用がある。環境にやさしく，安全性も高いとされ，操作性に優れた除染剤として適用できないか，現在検討中である。

（2）EasyDECON™DF200（INTELAGARD Inc.）

EasyDECON™DF200は水溶性の除染剤でサリン（GB），マスタード（HD），ソマン（GD），V剤（VX）等のC剤や農薬等有機毒生物化学剤及び毒性物質を迅速に無毒化できると言われている。毒性や刺激性が少なく，自然分解されることから，大量に使用しても環境には大きな影響を与えないと言われている。あらゆる金属表面での使用が可能で泡沫剤，液体スプレー，及び噴霧剤として使用できる。本剤は各種C剤を有効に無力化すると言われ，炭疽菌を始めとした生物剤についても増殖を完全に抑制する

表5．生物剤に対する除染剤

種類	適用対象	使用法	使用上の注意事項
クレゾール石鹸	・汚染物 ・手足	3％水溶液として使用。	糧食・水・食器類には使用できない。
石炭酸	・被覆，寝具		
アルコール	・手指，皮膚 ・小型器具 ・レンズ類	エチルアルコール　70—80％ イソプロピルアルコール　30—50％	イソプロピルアルコールは，エチルアルコールよりも除染力が強い。
ホルマリン	・汚染物 ・車両類 ・被覆，寝具	3％水溶液として使用。	皮膚，眼，鼻，喉を刺激する。
逆性石鹸	・手指 ・食器調理具	3％水溶液として使用。	弱酸性の石鹸。
過マンガン酸カリ溶液	・うがい用	0.01％水溶液として使用。	飲み込まない。
さらし粉	・飲料水 ・野菜・果物 ・排泄物	5％水溶液として使用	野菜，果物は消毒後，よく水洗いする。

文献7より引用，改変。

ことが出来るとされる。本剤はすでに商品化（INTELAGARD Inc., CO, USA)され，米国の対テロ分野を担う各機関に配備されている。

（3）FAST-ACT（NanoScale Materials, Inc.）

FAST-ACTは，米国で開発された有害化学物質処理剤で，一剤で多品種の有毒物質の効果があると言われている。二酸化チタン及び酸化マグネシウムを原材料とした無毒性，速効反応性のナノ材料配合製品であり，広い範囲の有害化学薬品を無害化するのに有効で，化学剤も無力化する能力があるとされている。本剤もすでに商品化され，米国の対テロ分野を担う各機関や毒物を扱う事業所に配備されている。

（4）酸化チタンナノ粒子を用いた光触媒技術

光接触技術はわが国の得意とする基盤技術であり，基礎的研究に加えて，環境・衛生分野への応用研究も盛んであり，光触媒技術・素材・装置がすでに市場化され，普及している[8]。本研究では，毒性は低く，比較的安価である酸化チタンナノ粒子を光触媒素材として適用し，化学剤除染システムの構築を提案し，試作品の調製を開始している。酸化チタンは紫外線等光触媒により，水や酸素と反応し，強力な酸化力を有する活性酸素を発生し，有毒物質を酸化・無毒化するとされている（図2）。我々は酸化チタンナノ粒子を多孔性無機担体に包埋させ線維と組み合わせた除染剤を試作，化学剤（毒物）除染への適用の可能性を検証している。これらの技術は，現場の活用する資機材（防護服・防護マスク等）に適用して，自然除染作用を付加させ，より軽量・安全で高性能な装備品として開発していく。加えて，現場で紫外線を照射する条件下で生物化学剤を効率よく除去するシステムを設計し，救護所等で用いる空気清浄装置，自走式除染ロボットなどの試作（図3）を検討する。

（5）銀ナノ粒子／キトサン複合体を用いた新規生物化学剤除染材料

我々は易溶解性ガラスに硝酸銀を含有させその粉体（粒径10－100μM）を調製した。その粉体を生理食塩水に入れて，還元剤及び安定剤としてグルコースを添加したコロイド溶液を120℃，20分，5気圧でオートクレーブすることで安価，高収率で5－15nmの均一な銀ナノ粒子を生成できることを見出している（図4）[9]。この銀ナノ粒子はキトサン線維に強く吸着し，

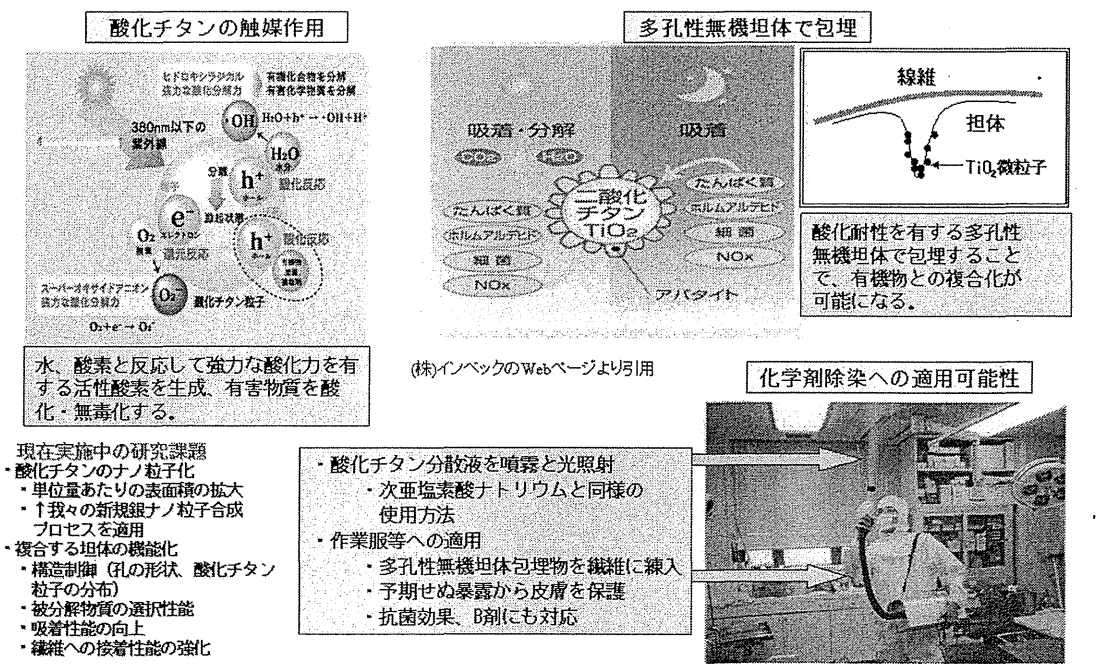

図2．酸化チタンの光触媒機能を利用した除染システム
酸化チタンは，光触媒反応により各種有機毒を分解・無力化する。
一部文献8より引用，改変。

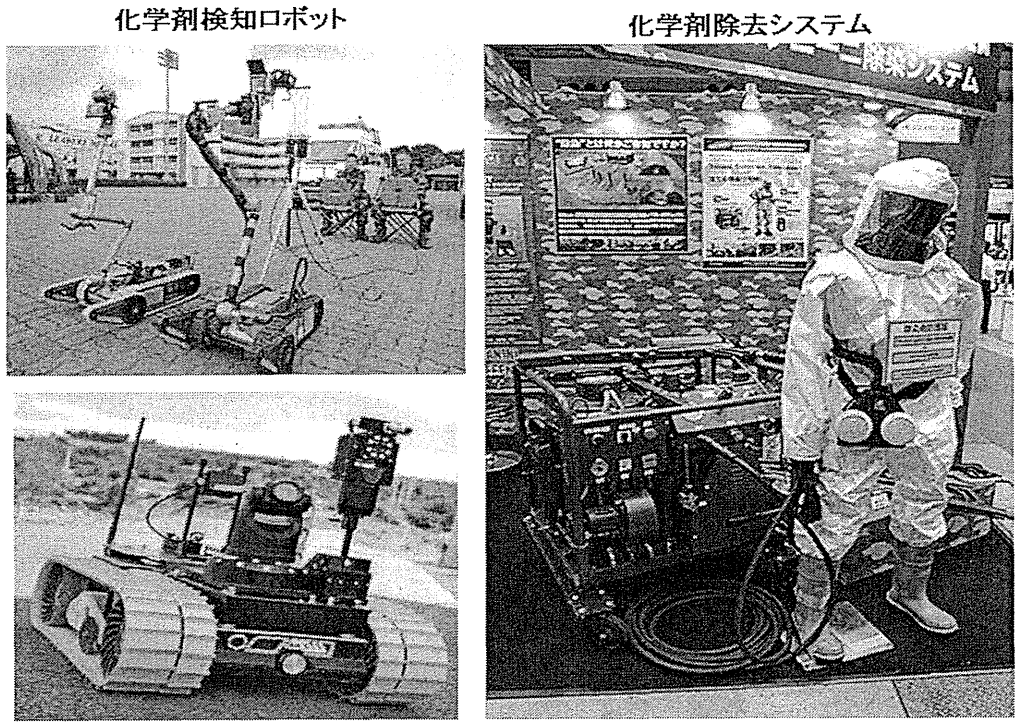

図3．検知・除染ロボットシステム
現在，実用化されている検知・除染用ロボットのイメージを示す。
一部文献7より引用，改変。

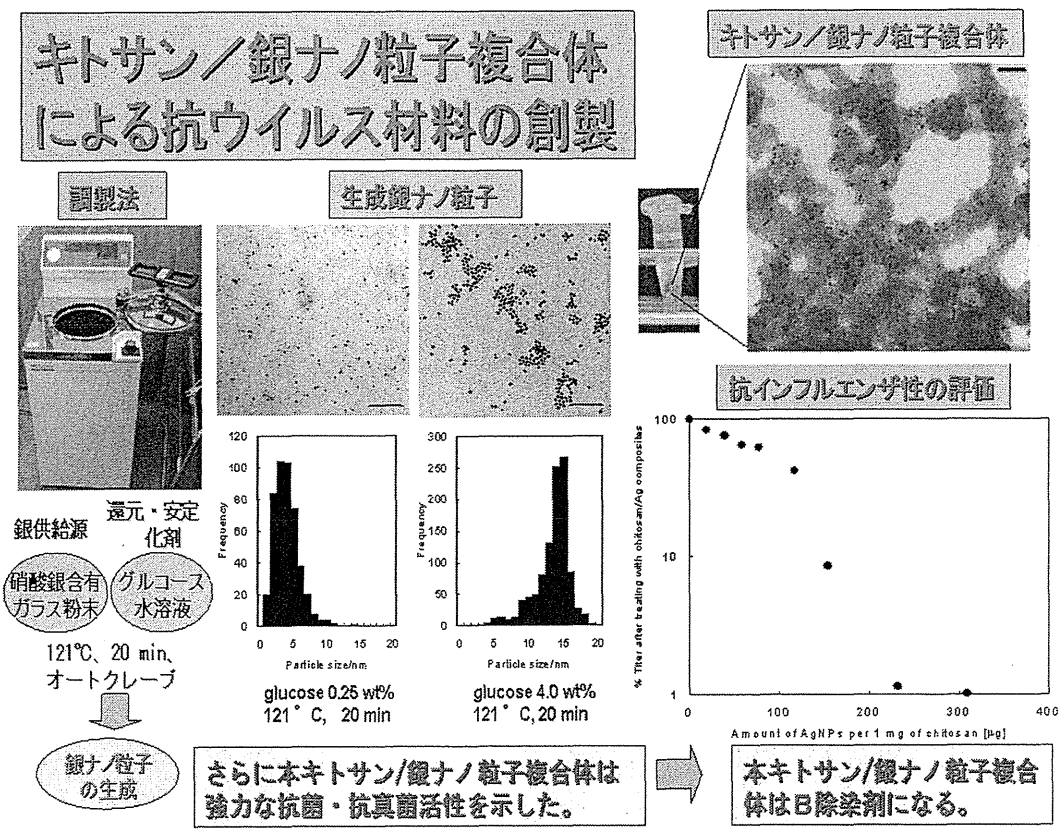

図4．キトサン／銀ナノ粒子複合体の抗ウィルス活性
一部文献9より引用，改変。

銀ナノ粒子／キトサン複合体を形成する。この新規銀ナノ粒子／キトサン複合体には抗真菌や抗菌性のみでなくインフルエンザ等の強い抗ウィルス活性や防臭性が見出されている。我々は，生物化学剤の除去のためキトサンを適用した新規多孔性吸着剤の開発を進めているが，これに銀ナノ粒子を包含させることで，有機物である化学剤及びウィルスを含んだ生物剤両者を除染できるマテリアルの開発を進めている。

（6）微生物を用いた膜分離活性汚泥式洗浄・排水処理システムの化学剤・放射性物質除染への適用[10]

化学剤，農薬，放射性物質，ダイオキシン，砒素，有機金属，環境ホルモン等，汚染が広がっている。これらは単なる安全保障上の要請だけではなく，地球環境の問題であり，一刻も早い適切な対策が必要である。ここで紹介する膜分離活性汚泥式洗浄排水処理設備は，自然界における自浄作用を利用することにより，省ランニングコストを実現したもので，タンクとポンプを主体とする構成で加圧，昇温の必要もなく安全かつ簡便な設備となっている。二次廃棄物発生量も少なく，水質浄化，毒物及び放射性物質除去性能を有している。活性汚泥は，主としてバクテリア，真菌などから構成されている微生物群集であり，活性汚泥法とはこれら微生物群集の代謝機能を利用して排水を浄化する方法である。本システムでは浄化槽内に分離膜を設置し，従来の標準活性汚泥法の浄化槽と沈降分離槽を一つの膜分離浄化槽に統合しており，装置のコンパクト化と膜を通してろ過するため，曝気槽内の汚泥濃度を高く維持することが可能となり，処理能力も高い（図5）。さらに本システムの主な二次廃棄物発生源は有機物を分解

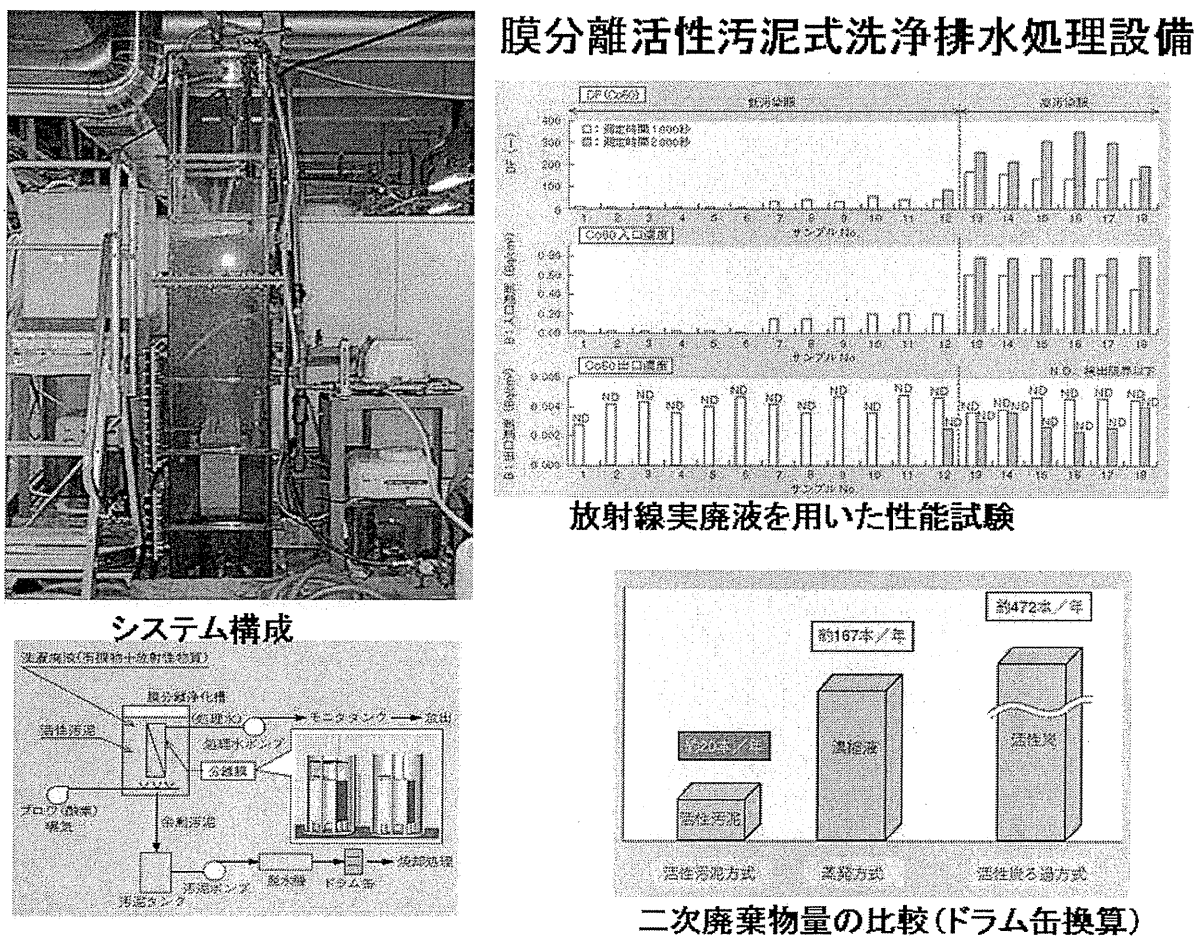

図5．膜分離活性汚泥式洗浄排水処理システムと能力
本システムにより，効果的に汚染水や汚泥を濃縮・除染できる。
一部文献11より引用，改

することによる活性汚泥の増加分のみであり，二次廃棄物発生量の低減も実現している。

おわりに

生物化学剤が用いられるテロの現場において，有害・猛毒であり種類が多岐にわたる生物化学剤を，安全・効率的，効果的に除去することが被害の拡大防止，現場の復旧に求められている。現状で行なわれている生物化学剤の除去方法は十分とは決して言えない。問題点としては，1）方法と効果の科学的検証が不十分で，科学的データがない，2）次亜塩素酸塩などの除染剤は毒性が高くしかも環境への負担が大きい，3）すべての生物化学剤に有効ではなく，長持続性・高抵抗性の剤が存在する，4）持続性が低く，その効果は一過性に過ぎない，5）大量の洗浄排水が発生しその処理は困難である，などがあげられる[11]。テロ現場での除染ニーズは，ヒト・環境に安全で，剤全般に有効で，効率的・持続性があり，それらが科学的データによって十分に検証されており，洗浄排水の心配が少ないものである。我々は現在，生物化学剤の除染のためハイパー・イオン水の適用，新規吸着性材料として銀ナノ粒子／キトサン複合体の適用，そして光触媒技術として酸化チタンナノ粒子の応用の可能性を検証し，生物化学剤除染のための上記目的達成に向けて研究を推進している。さらにバクテリア（活性汚泥）を適用する化学剤，有機毒，及び放射性物質洗浄除去のためのシステムとして，膜分離活性汚泥式洗浄・排水処理設備について記述した。

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Clearance technology of biological and chemical agents

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Abstract: In a field of terrorism used a wide variety of biological and chemical agents which are harmful and deadly poisonous, it is important to effectively and efficiently clear those agents for prevention of expanded damages and retrievals from the damage. The needs for their clearances in the disaster areas are safe for human body and easy on the environments as well as efficiencies, endurances and versatilities for those clearances. Furthermore, they are required evaluations based on scientific data. We are currently studying on applications of hyper-ion water, nano-silver particles/chitosan complexes as a functional absorbent and nano-titanium oxides as an optical catalyst for clearances of biological and chemical agents. We also introduce in this review a membrane-separation system using bioactive muddiness for drainage and irrigation.

Key words: Clearance of biological and chemical agents ／ nano-silver particles/chitosan complexes ／ functional absorbent ／ nano-titanium oxides ／ optical catalyst.

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Fragmin/protamine microparticles to adsorb and protect HGF and to function as local HGF carriers in vivo

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ABSTRACT

The clinical efficacy of hepatocyte growth factor (HGF) in tissue repair can be greatly enhanced by high affinity, biocompatible drug carriers that maintain the bioactivity and regulate release at the target site. We produced 0.5–3.0 μm fragmin (low molecular weight heparin)/protamine microparticles (F/P MPs) as carriers for the controlled release of HGF. F/P MPs immobilized more than 3 μg of HGF per mg of MPs and gradually released the absorbed HGF into the medium with a half-release time of approximately 5 days. Compared with HGF alone, HGF-containing F/P MPs substantially enhanced the mitogenic effect of HGF on cultured human microvascular endothelial cells, by prolonging the biological half-life, and its conjugation to F/P MPs protected HGF from heat and proteolytic inactivation. F/P MPs disappeared 8 days after subcutaneous injection in mice, suggesting that they are rapidly biodegraded. Furthermore, the number of large (diameter ⩾200 μm or containing ⩾100 erythrocytes) and medium (diameter 20–200 μm or containing 10–100 erythrocytes) lumen capillaries 8 days after injection of HGF-containing F/P MPs was significantly higher than that after injection of HGF or F/P MPs alone. Furthermore, the number of small (diameter ⩽20 μm or containing 1–10 erythrocytes) lumen capillaries was significantly higher 4 days after injection of HGF-containing F/P MPs. This increased angiogenic activity of HGF in vivo is probably due to both sustained local release and protection against biodegradation by the F/P MPs. Thus, F/P MPs may be useful and safe HGF carriers that facilitate cell proliferation and vascularization at sites of tissue damage.

1. Introduction

Preclinical studies have demonstrated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia [1,2]. Although growth factors such as hepatocyte growth factor (HGF) [3], fibroblast growth factor-2 (FGF-2) [4], and vascular endothelial growth factor [5] can induce neovascularization in ischemic models, this strategy has not always been successful [6] because of the high diffusibility and short biological half-life of these growth factors in vivo. Therefore, modifications that locally retain and prolong biological activity in vivo may enhance the efficacy of trophic and angiogenic factors for tissue regeneration and vascularization.

HGF was first identified as a potent mitogen for mature hepatocytes, and the gene was subsequently cloned [6]. Injection of HGF prevented endotoxin-induced hepatic failure in mice with fulminant hepatitis by inhibiting apoptosis [7], and HGF gene therapy improved the survival rate of rats with lethal liver cirrhosis [8]. HGF stimulated the growth of endothelial cells without stimulating the replication of vascular smooth muscle cells, indicating that it is a potential angiogenic growth factor [2,9,10]. Furthermore, HGF is neurotrophic to hippocampal, cortical, midbrain dopaminergic, cerebellar granular, sensory, motor, and sympathetic neurons and neuroblasts [11].

HGF binds with high affinity to heparinoids (heparin, heparan sulfate, and other heparin-like molecules), and this heparinoid binding could help maintain its biological stability and mitogenic activity [12,13]. HGF is synthesized as a biologically inactive precursor that is proteolytically cleaved to give a disulfide bonded heterodimer [14]. Two alternative transcripts have also been

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identified that encode truncated variants of HGF, and these smaller isoforms, designated HGF/NK1 and HGF/NK2, behave as antagonists or partial agonists depending on the assay conditions [15]. All these HGF isoforms bind to the tyrosine kinase transmembrane HGF receptor MET and heparinoids [12].

HGF markedly accelerates regeneration of damaged organs in animals with hepatic and renal failure. However, since its half-life is only approximately 5 min because of rapid diffusion and clearance by the liver, its local biological activity may be limited in vivo [16,17]. This rapid clearance necessitates very large doses (>100 $\mu g\ kg^{-1}$) to exert hepatotrophic effects in vivo [18]. Thus, the development of a reliable, efficient and safe drug delivery system for HGF may facilitate potential clinical applications in tissue regeneration and vascularization.

We previously developed a photocrosslinked chitosan hydrogel [19] and a 6-O-desulfated heparin hydrogel [20] for the controlled release of growth factors such as FGF-2, and we demonstrated that these hydrogels served as efficient carriers for the local administration of a subcutaneous or muscular injection facilitating neovascularization and granulation tissue formation at wound sites in vivo [19,20]. 6-O-Desulfated heparin hydrogels [20] are not readily injectable, while photocrosslinkable chitosan hydrogels with modified heparin and FGF-2 are. However, thick needles ($\geqslant$20 G) are required to inject these hydrogels, and these needles sometimes become clogged with the hydrogel [19]. Therefore, these hydrogels have not been evaluated as possible HGF carriers.

Heparin and low molecular weight heparin (fragmin) are known to interact with various proteins, including HGF, suggesting that a heparin-based carrier could be useful as a therapeutic agent to locally retain and prolong HGF bioactivity at the injury site. While high doses of heparin carry a high risk of bleeding [21], fragmin has several pharmacological and practical advantages. The lower protein binding affinity of fragmin produces a lower and more predictable anticoagulant response, thereby obviating the need for laboratory monitoring to adjust the dosage [21]. In addition, one or two subcutaneous injections per day are sufficient to maintain therapeutic concentrations because of the longer plasma half-life of fragmin [21]. Protamine, a purified mixture of proteins obtained from fish sperm, neutralizes heparin and fragmin by forming a stable complex that lacks anticoagulant activity [22]. It is used clinically to reverse the anticoagulant activity of heparin following cardiopulmonary bypass and in cases of heparin-induced bleeding [23].

We previously reported that fragmin/protamine microparticles (F/P MPs) are injectable carriers for the controlled release of FGF-2 [24]. A mixture of fragmin and protamine yields water insoluble microparticles (approximately 0.5–3 μm in diameter). FGF-2-containing F/P MPs induced substantial vascularization and fibrous tissue formation in vivo [24]. In the present study we examined the utility of F/P MPs as carriers for the controlled release of HGF. F/P MPs have a high affinity for HGF, enhance HGF mitogenic activity in vitro, and protect HGF from heat and proteolytic inactivation. Furthermore, HGF-containing F/P MPs are more effective in inducing angiogenesis in vivo than HGF and F/P MPs alone.

2. Materials and methods

2.1. Preparation of HGF-containing F/P MPs

F/P MPs were prepared as described previously [24]. In brief, 0.3 ml of protamine solution (10 $mg\ ml^{-1}$, Mochida Pharmaceutical Co., Tokyo, Japan) was added dropwise to 0.7 ml of fragmin solution (6.4 $mg\ ml^{-1}$, Kissei Pharmaceutical Co., Tokyo, Japan) and vortexed vigorously for approximately 2 min. In this study, to maximize the production of MPs, protamine and fragmin were mixed at a ratio of 3:7 by volume. To remove unreacted reagents the mixture was centrifuged at 8000 r.p.m. for 10 min at 4 °C (MX-300, Tomy, Tokyo, Japan), and the pelleted MPs (approximately 0.5–3 μm in diameter) were resuspended in 1 ml of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Oriental, Tokyo, Japan). The diameter range of the F/P MPs was determined by comparison with the scale bars of an optical microscope. This mixture yielded approximately 5 mg of F/P MPs (dry weight). The F/P MPs did not aggregate at 5 $mg\ ml^{-1}$, hence 5 $mg\ ml^{-1}$ was selected as the standard stock concentration. To prepare HGF-containing F/P MPs for in vivo muscular injection

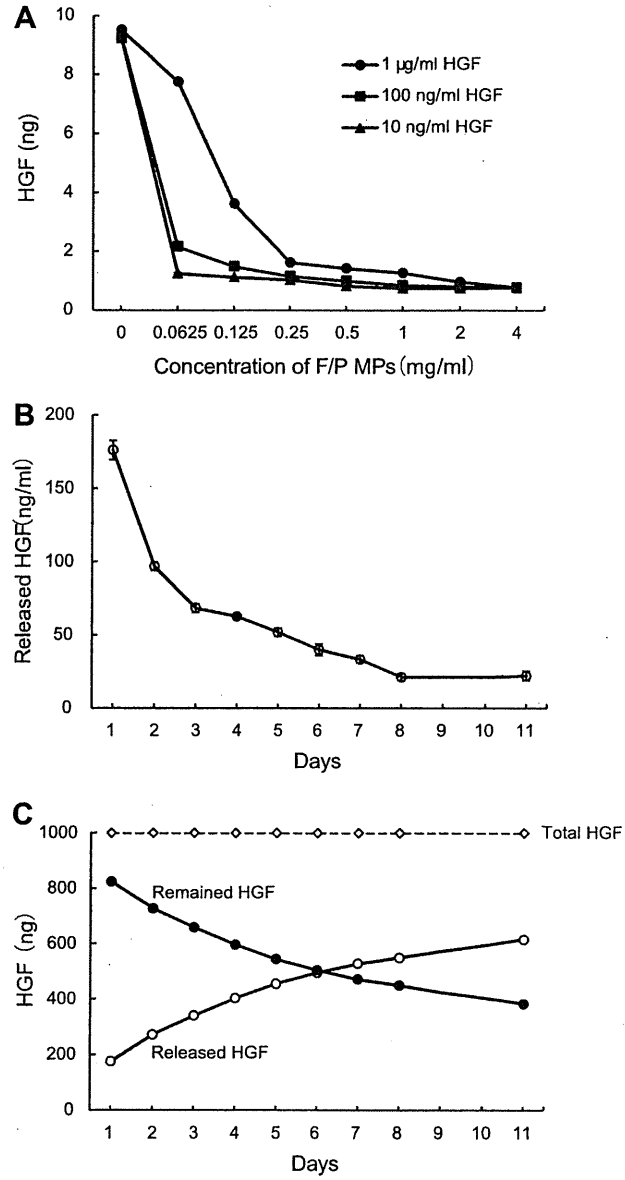

Fig. 1. (A) Absorption of HGF onto F/P MPs. The indicated amounts of F/P MPs were added to DMEM containing FBS and HGF. The solution was centrifuged to precipitate HGF-containing F/P MPs, and HGF remaining in the supernatant was measured by ELISA. (B) HGF release into DMEM. HGF release into DMEM on a given day from HGF-containing F/P MPs was quantified by ELISA of the supernatant. (C) Cumulative HGF release and retention. HGF initially added to the F/P MP solution was defined as 100%. The amount of HGF released into DMEM or retained on HGF-containing F/P MPs was calculated. Each data point represents the mean of three independent measurements.

recombinant human HGF (R&D Systems Inc., Minneapolis, MN) (5 μg ml^{-1}) in DMEM without heat-inactivated fetal bovine serum (FBS) was added to F/P MPs (5 mg ml^{-1}) on ice, mixed by vortexing, and incubated for 18 h at 4 °C on a rotary shaker. Under these conditions almost all of the HGF is bound to and immobilized on F/P MPs.

2.2. Evaluation of HGF binding by ELISA

Solutions of F/P MPs at 0–4 mg ml^{-1} were prepared in DMEM containing 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, and 2% FBS. HGF (10, 100 or 1000 ng ml^{-1} in F/P MP solution) was added and the mixture was incubated for 18 h at 4 °C on a rotary shaker. The mixture was then centrifuged at 8000 r.p.m. for 10 min to remove the precipitated HGF-containing F/P MPs. The amount of HGF remaining in the supernatant was estimated by measuring the absorbance at 450 nm using a microplate reader and a Quantikine Human HGF Immunoassay Kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

ELISA was also performed to evaluate HGF release from HGF-containing F/P MPs. In brief, HGF-containing F/P MPs were incubated in DMEM (2 ml) containing 2% FBS and antibiotics at 37 °C for 10 days with gentle shaking. DMEM was removed daily for 10 days and centrifuged at 8000 r.p.m. for 10 min to obtain supernatants. The amount of HGF in the supernatant was measured by ELISA. The total amount of HGF released on a given day was the sum of all previous supernatant HGF concentrations.

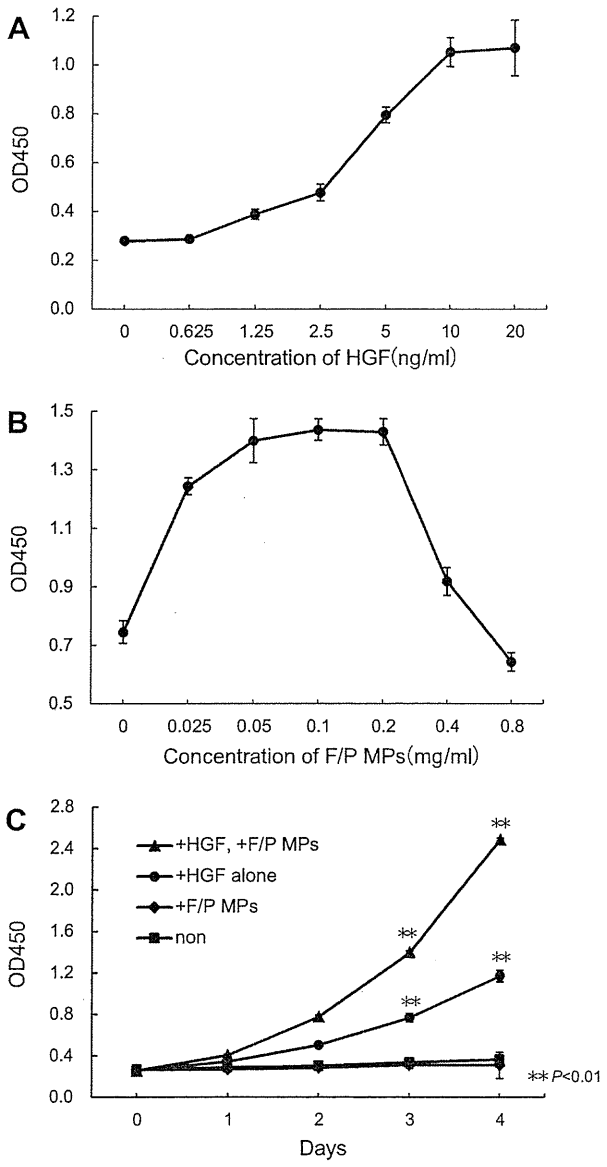

Fig. 2. (A) Influence of HGF concentration on MVEC growth in DMEM containing 5% FBS without F/P MPs. (B) Influence of F/P MP concentration on MVEC growth in DMEM containing FBS with HGF (5 ng ml^{-1}). (C) Influence of F/P MPs (0.1 mg ml^{-1}) and HGF on MVEC growth in DMEM containing FBS. Each data point represents the mean ± SD values from four determinations. An unpaired *t*-test was used to compare the experimental and control groups. $^{**}P < 0.01$.

2.3. Mitogenic activity of HGF alone and HGF-containing F/P MPs on human microvascular endothelial cells (MVECs)

MVECs (Takara Biochemical Corp., Otsu, Japan) were cultured in DMEM containing 5% FBS, 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, and the indicated concentrations of HGF and/or F/P MPs. The cells used in these studies were all between cell cycle passages four and eight. MVECs were seeded at an initial density of 3000 cells per well in 96-well tissue culture plates and grown for 3 days in 200 μl of DMEM containing 5% FBS, 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, and either HGF alone, HGF and F/P MPs, or F/P MPs alone at the indicated concentrations. After incubation the medium was removed and 100 μl of fresh medium containing 10 μl of WST-1 reagent (Cell Counting Kit, Dojindo Co. Ltd, Kumamoto, Japan) was added to each well. The cell number was estimated by measuring the absorbance at 450 nm using a microplate reader.

2.4. Protection of HGF from inactivation by F/P MPs

To determine whether binding to F/P MPs can sustain HGF activity, 100 ng of HGF was added to 1 ml of DMEM containing

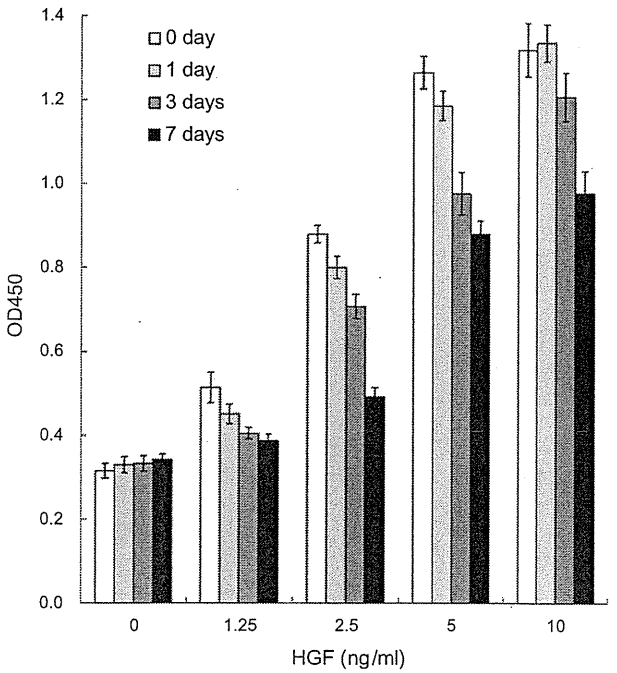

Fig. 3. The stability of pre-immobilized HGF as determined by MVEC proliferation assay. F/P MPs were incubated with HGF. After incubating the HGF-containing F/P MP stock solution for the indicated time periods MVECs were cultured in DMEM containing FBS and diluted pre-immobilized HGF-containing F/P MPs for 3 days. Data represent the means ± SD of four determinations.

2% FBS or to DMEM containing 1.0 mg ml^{-1} F/P MPs and 2% FBS. These stock solutions were incubated at 37 °C for 0, 2, 4 and 7 days and then 1 ml of the diluted HGF (nominally 20, 10, 5, 2.5 and 0 ng ml^{-1}) and F/P MPs (200, 100, 50, 25 and 0 μg ml^{-1}, respectively) in DMEM containing 5% FBS was used in MVEC cultures. Again, cell proliferation was assayed after 3 days using the WST-1 reagent.

To examine if F/P MPs protect HGF against heat inactivation, 200 μl stock solutions of either 100 ng HGF-containing F/P MPs (1 mg ml^{-1}) or HGF alone (100 ng ml^{-1}) in DMEM without FBS were heated at 37, 42, 47, 52, 57, 62, 67 and 72 °C for 20 min. To determine if F/P MPs protected against proteolytic cleavage by trypsin, 100 μl of trypsin–EDTA solution (0.5 mg ml^{-1} trypsin, 0.2 mg ml^{-1} EDTA/4Na^{+} in HBSS, Sigma, St Louis, MO) was added to 100 μl of each chilled stock solution (HGF alone or HGF-containing F/P MPs DMEM without FBS) and incubated at 37 °C for the indicated periods of time (10–120 min). After incubation, 100 μl of FBS was added to each trypsinized stock solution to stop the trypsinization reaction. These heat-treated or trypsinized stock solution were diluted 10-fold with DMEM containing 5% FBS. MVEC were cultured in the prepared media for 3 days, and the cell number measured as described above.

2.5. Vascularization induced by HGF-containing F/P MPs

Animal experiments were performed according to the protocol approved by the Animal Experimentation Committee of the National Defense Medical College (Saitama, Japan). Male C57 BL/6 mice (6–7 weeks old) were purchased from Clea Japan Inc. (Tokyo, Japan). To prepare HGF-containing F/P MPs for in vivo injection 5 μg HGF and 5 mg F/P MPs were dissolved in 1 ml of DMEM and the solution vortexed. Next, 200 μl of HGF-containing (1 μg) F/P MPs (1 mg) or HGF alone (1 μg) was carefully injected into the right and left sides of the back subcutis of mice 2 cm above the tail root. To evaluate neovascularization mice were killed 4, 8, 11 or 15 days after injection. The excised skin tissue around the injection site was fixed in 10% formaldehyde solution (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin, and sectioned at 4 μm. The sections (~10 × 1.0 mm) were mounted on glass slides and stained with hematoxylin and eosin (H&E). The microscopic

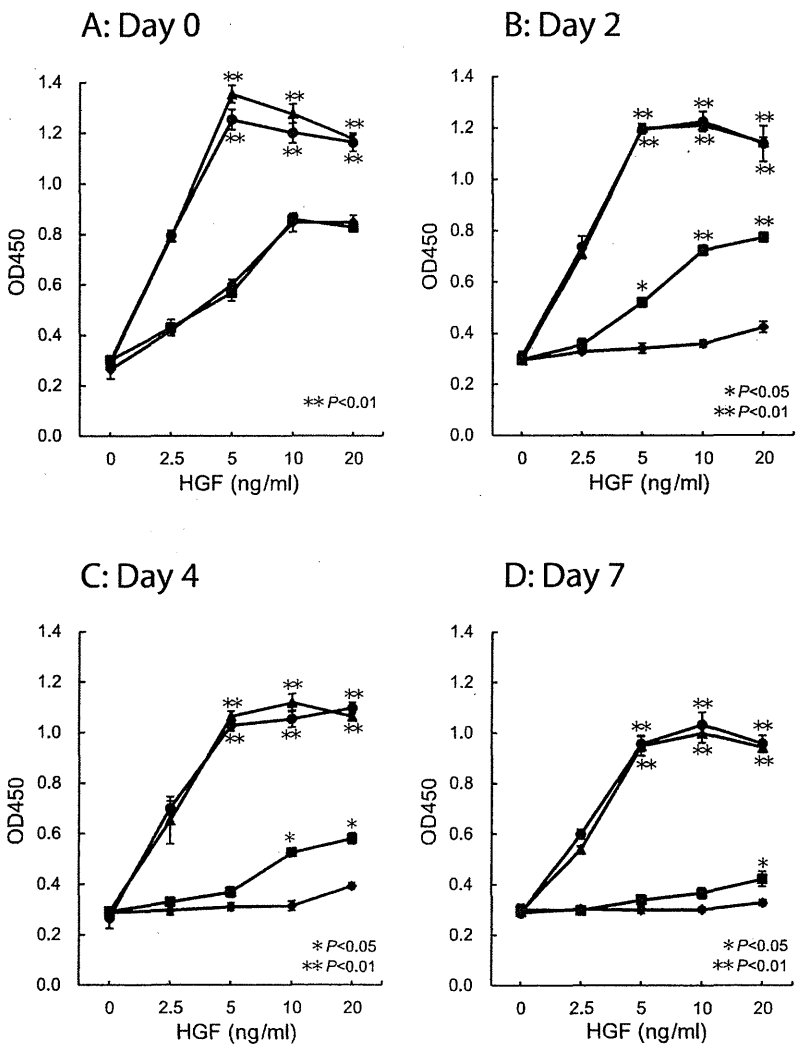

Fig. 4. The protective effect of F/P MPs on HGF. Stock solutions of HGF-containing F/P MPs with (●) or without (▲) FBS and HGF alone with (■) or without (♦) FBS were incubated for (A) 0, (B) 2, (C) 4, and (D) 7 days. Stock solutions were diluted to the indicated nominal concentrations of HGF-containing F/P MPs with DMEM. MVECs were cultured for 3 days using one of the prepared media. Data represent the means ± SD of four independent determinations. An unpaired *t*-test was used to compare the experimental and control groups. $^{**}P < 0.01$, $^{*}P < 0.05$.

field (100×) showing the largest capillary density in each section was photographed, and the number of large (diameter ⩾200 μm or containing ⩾100 erythrocytes), medium (diameter = 20–200 μm or containing 10–100 erythrocytes) and small (diameter ⩽20 μm or containing 1–10 erythrocytes) lumen capillaries were counted.

2.6. Statistical analyses

Group means were compared by an unpaired Student's *t*-test using Stat Mate III for Windows (ATMS Co., Tokyo, Japan). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Binding of HGF to F/P MPs

To assess the binding affinity of F/P MPs for HGF various concentrations of F/P MPs were added to 10, 100, or 1000 ng ml^{-1} HGF solution. The solutions were centrifuged to precipitate HGF-containing F/P MPs, and HGF remaining in the supernatant was measured by ELISA (Fig. 1A). The concentration of HGF was progressively reduced by increasing the F/P MP concentration. As little as 0.5 mg ml^{-1} F/P MPs reduced the remaining free HGF to near the limit of detection by ELISA, indicating that the majority of HGF molecules were bound to F/P MPs and subsequently precipitated by centrifugation. A F/P MPs concentration of 0.25 mg ml^{-1} adsorbed about 84% of 1 μg ml^{-1} HGF in DMEM containing 2% FBS, indicating that 1 mg of F/P MPs can adsorb approximately 3.3 μg of HGF. Although various proteins in FBS, especially heparin-binding proteins, appear to interact with F/P MPs, fetal bovine albumin does not interact with F/P MPs. Therefore, it is speculated that HGF may specifically interact with F/P MPs even in the presence of FBS in vitro.

Approximately 19% of the HGF from HGF-containing F/P MPs (prepared by 18 h incubation at 4 °C) was released into the medium within 1 day (Fig. 1B and C), while approximately 43% and 38% remained in the F/P MP fraction after 7 and 10 days, respectively (Fig. 1C). Thus, the immobilized HGF was gradually released into the medium with a half-release time of approximately 5 days. Dissolution of F/P MPs in vitro was not observed visually after 2 weeks.

3.2. Binding of HGF to F/P MPs enhanced and prolonged HGF mitogenic activity in MVEC cultures

Incubation with HGF at 1.25–20 ng ml^{-1} stimulated the proliferation of MVECs, with a half-maximal effect at approximately 5 ng ml^{-1} (Fig. 2A). Addition of F/P MPs (50–200 μg ml^{-1}) and 5 ng ml^{-1} HGF to DMEM containing 5% FBS significantly enhanced the proliferation of MVECs (Fig. 2B). Indeed, the mitogenic effect of 5 ng ml^{-1} HGF was near maximal at an F/P MP concentration of 100 μg ml^{-1} (Fig. 2B). The combination of 5 ng ml^{-1} HGF plus 100 μg ml^{-1} F/P MPs approximately doubled the cell number on day 3 in culture compared with cells grown in the presence of HGF alone (Fig. 2C) and reduced the cell doubling time over days 1–3 to approximately 25 h from 47 h in HGF alone.

The growth of MVECs was stimulated in a concentration-dependent manner by the diluted pre-immobilized HGF-containing F/P MPs (Fig. 3), indicating that immobilized HGF retained significant bioactivity even after 7 days at 37 °C. This result suggests that HGF mitogenic activity was stabilized when HGFs were pre-immobilized on F/P MPs.

3.3. F/P MPs prolonged the half-life of HGF bioactivity and protected against heat and proteolytic inactivation

When 100 ng ml^{-1} HGF was incubated for 2 days or more in DMEM with or without 2% FBS at 37 °C the mitogenic activity was substantially reduced (<90% by day 7, Fig. 4A–D). However, no decrease in mitogenic activity was observed if 100 ng ml^{-1} HGF was incubated in the presence of 1 mg ml^{-1} F/P MPs (either with or without 2% FBS) for 7 days (Fig. 4D). The biological half-life of 10 ng ml^{-1} HGF in DMEM containing 5% FBS was only about 3 days in the absence of F/P MPs (Fig. 4B), but more than 7 days in the presence of F/P MPs (Fig. 4D).

To determine whether F/P MPs could protect HGF bioactivity against heat inactivation HGF (100 ng ml^{-1}) was heated with or without F/P MPs (1 mg ml^{-1}). Heating HGF above 42 °C resulted in a reduction in mitogenic activity both in the presence and absence of F/P MPs (Fig. 5A), but the temperature at which half of the mitogenic activity was lost was substantially higher (56 °C) in the presence of F/P MPs than in the absence of F/P MPs (45 °C). Thus, F/P MPs can protect HGF from heat inactivation. Similarly, F/P MPs protected HGF against trypsin-mediated proteolysis

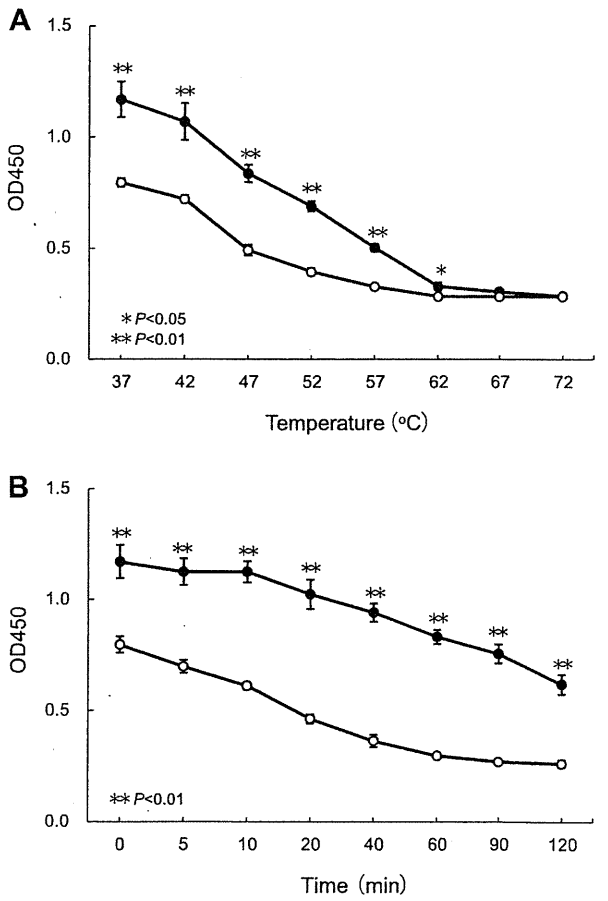

Fig. 5. The protective effect of F/P MPs on the bioactivity of (A) heat-treated and (B) trypsin-treated HGF. (A) The stock solutions (●) and HGF alone (○) were incubated at the indicated temperatures. The heated HGF solutions were diluted with DMEM containing FBS. MVECs were cultured using one of the prepared media. (B) DMEM stock solutions (●) and HGF alone (○) were treated with trypsin for the indicated times. After trypsinization proteolysis was stopped by adding FBS, the inactivated HGF in the stock solutions was diluted with DMEM containing FBS, and MVECs were cultured using one of the prepared media. Data points represent the means ± SD of four independent determinations. An unpaired *t*-test was used to compare the experimental and control groups. $^{**}P < 0.01$, $^{*}P < 0.05$.

(Fig. 5B). In the presence of trypsin the biological half-life of HGF was only 20 min, while that of HGF-containing F/P MPs was 120 min.

3.4. Vascularization induced by HGF-containing F/P MPs in vivo

To confirm the bioactivity of HGF-containing F/P MPs in vivo we injected mice with HGF alone or HGF-containing F/P MPs and examined vascularization around the injection sites by H&E staining. Representative micrographs of tissue sections around each injection site on day 8 after injection are shown in Fig. 6. In this study the injected F/P MPs were not detected visually at the injection sites on day 8, suggesting that they are rapidly biodegraded, but small paste-like aggregates were observed on day 4 (data not shown). Several large (diameter $\geqslant$200 μm or containing $\geqslant$100 erythrocytes), medium (diameter 20–200 μm or containing 10–100 erythrocytes), and small (diameter $\leqslant$20 μm or containing 1–10 erythrocytes) lumen capillaries were observed in the subcutaneous tissue of mice injected with HGF-containing F/P MPs. Only minor increases in the number of medium and small sized vessels were observed after injection of HGF or F/P MPs alone.

The number of large, medium, and small vessels around the injection sites (about a 10 × 1.0 mm area) was then quantified (Fig. 7). Representative micrographs of tissue around each injection site are shown 4, 8, 11, and 15 days after injection (Fig. 7). 8 days after injection the numbers of mature large (Fig. 7A), medium (Fig. 7B), and small vessels (Fig. 7C) were significantly higher ($P < 0.05$) in subcutaneous tissue injected with HGF-containing F/P MPs than in tissue injected with HGF or F/P MPs alone. A significantly larger number of small vessels was observed in tissue injected with HGF-containing F/P MPs as early as 4 days after injection (Fig. 7C).

4. Discussion

HGF can accelerate the regeneration of damaged tissue in animal models of hepatic and renal failure, but the local biological activity of exogenous HGF may be limited in vivo without an

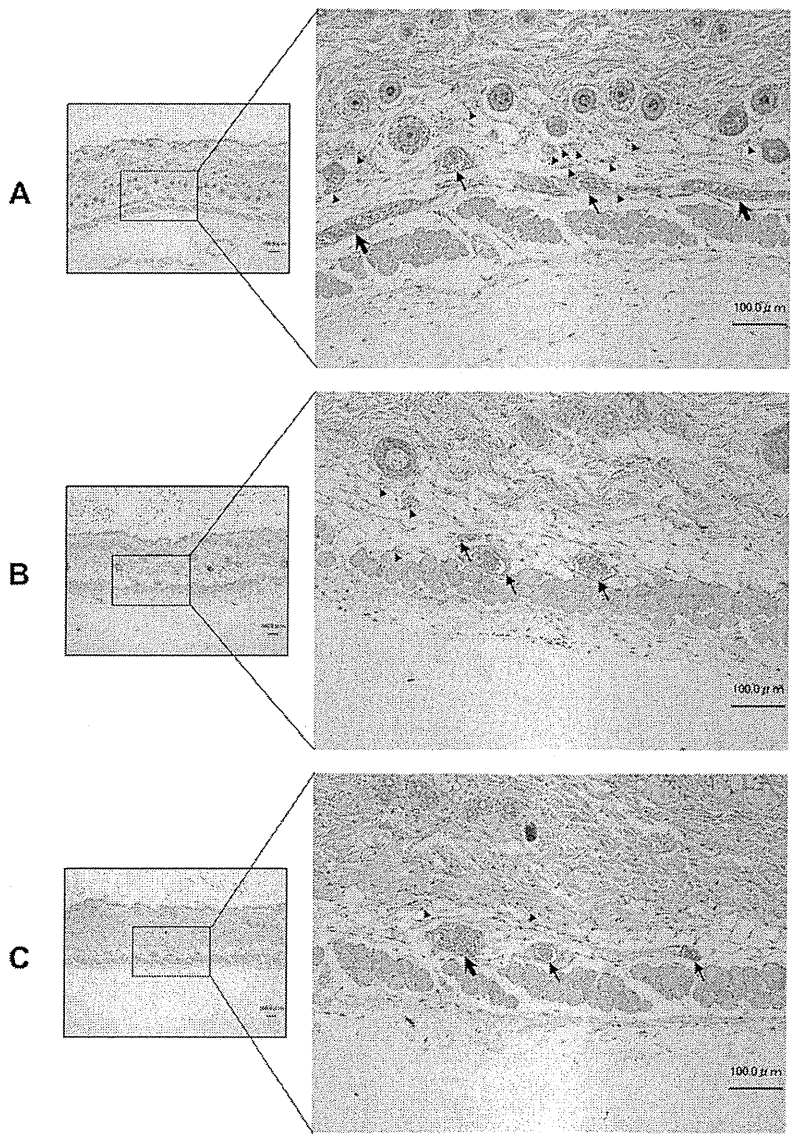

Fig. 6. Histological examination of subcutaneous sites 8 days after injection of (A) HGF-containing F/P MPs, (B) HGF alone, or (C) F/P MPs alone. Photographs are representative H&E stained tissue samples from 10 sites (1 per mouse) injected with either HGF-containing F/P MPs, HGF alone, or F/P MPs alone. The large arrows, small arrows, and triangles show large, medium, and small vessels containing erythrocytes, respectively.

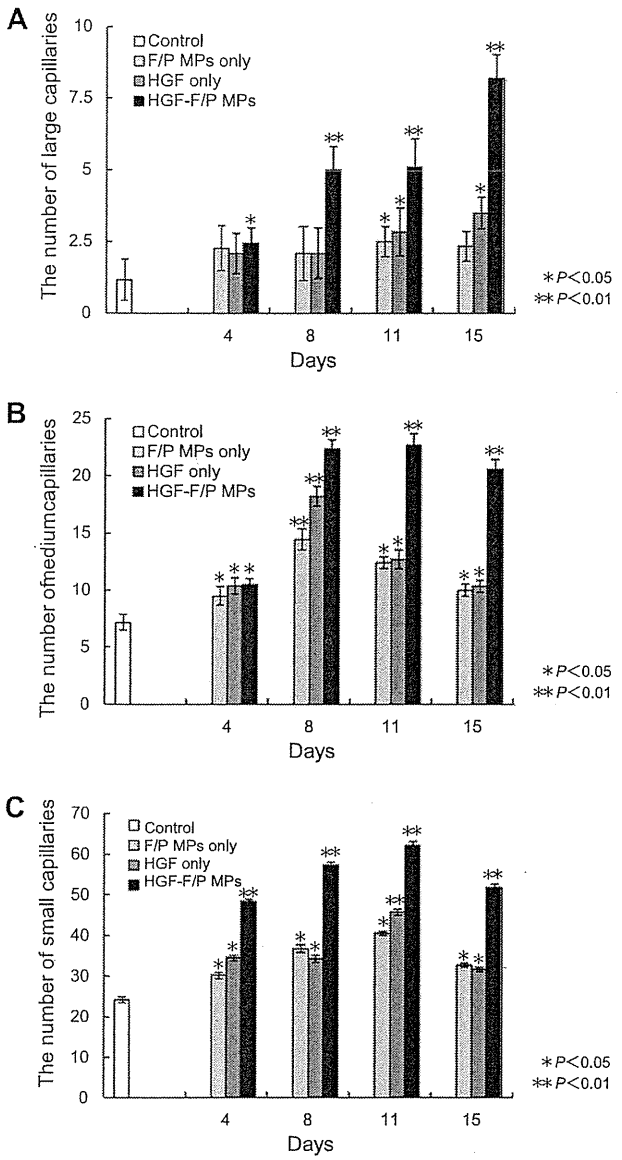

Fig. 7. Angiogenic effect of HGF-containing F/P MPs in vivo. The number of large, medium, and small capillaries in each sample (about 10 × 1.0 mm) was counted ($n = 10$). All values are means ± SD. An unpaired *t*-test was used to compare the experimental and control groups. $^{**}P < 0.01$, $^{*}P < 0.05$.

adequate protein delivery system that acts to maintain HGF at the target site and prevents rapid clearance by the liver [16,17]. Without such a system very large doses are usually required to exert a significant regenerative effect [18]. To enhance the repetitive efficacy of HGF we developed F/P MPs for high affinity absorption and controlled release of HGF. F/P MPs prolonged the biological activity of HGF and protected HGF against heat and proteolytic inactivation. Furthermore, these HGF-containing F/P MPs were injectable and biocompatible, and triggered significant angiogenesis at the site of injection. Thus, F/P MPs are a reliable, efficient and safe protein delivery system to enhance and stabilize HGF activity at local administration sites for subcutaneous or muscular injection.

Heparinoids and F/P MPs also prolong the biological half-life of FGF-2, and protect FGF-2 from heat and proteolytic inactivation [24–27]. Similarly, the present study has demonstrated that more than 3 μg of HGF bound to 1 mg of F/P MPs and was gradually released from HGF-containing F/P MPs in vitro. HGF-containing F/P MPs appeared to be bioactive, since they stimulated MVEC proliferation. In fact, HGF-containing F/P MPs were more mitogenic than HGF alone, reducing the cell doubling time from 47 to 25 h, possibly because bound HGF is more resistant to biodegradation and inactivation under physiological conditions. Indeed, proliferation of MVECs was substantially stimulated by preloaded F/P MPs even after 10 days at 37 °C, while HGF alone did not stimulate MVEC proliferation at all after 7 days preincubation at 37 °C. Furthermore, F/P MPs could effectively protect HGF against heat inactivation and trypsin degradation.

When HGF-containing F/P MPs were subcutaneously injected into the backs of mice large and medium vessels were induced near the injection site after 8 days. The number of small vessels induced by HGF-containing F/P MPs reached a maximum on days 8–11, after which a slight decrease in the rate of neovascularization occurred. No significant induction of large vessels was observed after injection of HGF or F/P MPs alone, although a slight induction of medium and small vessels was observed. We suggest that free HGF diffused away too rapidly to induce arteriogenesis and that inactivation of HGF remaining at the injection site within a few days also led to less efficient vascularization. The modest vascularization (mainly small and medium vessels) induced by F/P MPs alone may result from the binding of various endogenous angiogenic growth factors around the injection site, leading to local accumulation and controlled release.

It is known in polymer chemistry that positively and negatively charged polymers interact ionically [16,22]. Thus, basic protamine molecules complex with acidic fragmin molecules to form microparticles through ionic interactions. Our data indicate that bound polypeptides such as HGF are gradually released from F/P MPs with a half-release time of 5 days. Since F/P MPs are biodegradable in vivo, incorporating HGF on them will provide a controlled release system for the lifetime of the F/P MPs. Thus, F/P MPs provide an excellent biomaterial to immobilize, locally retain and gradually release HGF for optimal induction of neovascularization.

Fragmin enhanced HGF activity and HGF-induced tube formation by endothelial cells in vitro (data not shown). Protamine is also used clinically to neutralize heparin by forming a stable complex without anticoagulant activity [22]. The present study has demonstrated that F/P MPs strongly interact with HGF and that F/P MPs protect HGF from inactivation by heat and degradation by proteases. These HGF-containing F/P MPs bound to multiple cell surfaces [28], in addition to culture plates and biological matrices such as collagen [29]. Thus, HGF-containing F/P MPs would remain at the injection site and sustain a high local concentration of HGF.

Both chemical components of the F/P MPs are in clinical use and, hence, MPs may possess high clinical safety. It is thus proposed that HGF-containing F/P MPs may be a promising new controlled HGF release system to induce vascularization in ischemic limbs.

5. Conclusions

In the present study we evaluated the mitogenic effect of HGF-containing F/P MPs on cultured MVECs and the angiogenic effect in mice. Our main conclusions are (i) HGF is substantially adsorbed on F/P MPs, and in this state they are protected from inactivation, (ii) HGF is gradually released from HGF-containing F/P MPs through diffusion and/or biodegradation of the F/P MPs, and (iii) HGF-containing F/P MPs induce substantial vascularization in vivo. The results presented in this study indicate that F/P MPs may serve as an effective carrier for HGF, particularly for the local application of HGF at tissue injury sites.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Fig. 6, is difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.08.003.

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