⑤ サーベイランス

主な変更点	変更理由
疑似症定点サーベイランスらの実施 自動症候群サーベイランスの実施 疑い症例調査支援システムの活用 予防接種状況、副反応状況報告システムの実施	感染症法の改正 第5版ガイドラインでの同内容は抽象的な表現であったがその具体的な実施 厚生労働省のシステムとして整備されたため 必要性が認識されたため

⑥ 保健所における初期対応(疫学対応)

主な変更点	変更理由
保健所に置く各班の説明を組織体制の項から保健所の項に移した	保健所内部門の詳細な組織体制であり、関連記載のある部分に統一した。
要観察例などの用語を新型インフルエンザでの用語に準じて変更した	類似した健康危機に統一的な対応を取ることが望ましいため。
患者情報の入力・共有にはNESID疑い症例支援システムを用いることを基本としたが入力方法・時間など運用上の課題があると考えられ従来の紙様式も併記した。	現時点での実効性を担保し将来の方向を示すため紙媒体と電子システムと併用とした。

⑦ 検査に関すること

主な変更点	変更理由
検査材料の採取	変更なし
検体材料の輸送	変更なし

⑧ ワクチン接種対応に関すること

主な変更点	変更理由
Ⅱ天然痘ワクチン接種戦略	新規追加、具体的な接種戦略がなかったため基本方針(レベルの考え方)の方針変更に対応

レベルに応じたワクチン接種 レベルⅠ　ファーストレスポンダーⅠ レベルⅡ　ファーストレスポンダーⅡ レベルⅢ　リングワクチネーション レベルⅣ　マスワクチネーション	レベルⅠでは、最初の①例に対応するもののみ接種。 レベルⅡではファーストレスポンダーに広く接種 レベルⅢからⅣへの切り替えは、都道府県ごとであり、接触者把握のための積極的疫学調査のキャパシティーを超えた時点で転換。
リングワクチネーションに関しては、一定人口レベル（都道府県、大都市）に接種会場を設けるとともに、必要に応じて巡回接種の出来る体制を整える	・ リングワクチネーションが実施される際には、ファーストレスポンダーへの接種と同時期に行われる可能性がある。（国内発生時においては同時期になる可能性が高い） ・ ワクチン接種を行う医師の確保には限界がある。効率的な運用を余儀なくされる。 ・ ワクチンを効率的に活用するためには、一度開封したワクチンでできるだけ多くの接種を行う方がよい。 ・ 自宅などから移動できない患者も想定する必要がある。
マスワクチネーションについては、小学校レベルで接種会場を設ける	アクセスと効率性
ワクチンの輸送 ・ 地域によっては、主要空港まで航空機、ヘリで輸送 ・ 主要空港または県の施設（衛生研究所等）には保管機能 ・ 主要空港または県の施設（衛生研究所等）から保健所等までの輸送は都道府県が実施（県警、自衛隊、運送会社等を活用）	備蓄は安全性も考え国が実施する。 都道府県までの輸送は国が実施。 都道府県内の輸送は都道府県が実施 温度管理が可能な方法での輸送が必要 冷凍輸送車（-20℃）で輸送できない可能性もある。 現行法では他職種での接種は困難
ワクチン輸送時の温度管理 ワクチン接種の要員確保	接種者には熟練は特に必要ないが、接種会場の責任者（助言者）は熟練者がいることとされた。熟練者は、ワクチンの接種と会場のマネージメントに熟練する必要である。

医師が行う 国で、平時より最低全都道府県に同時に赴ける人数を確保し、その上で、ファーストレスポンダーⅡの接種を通して熟練者を拡大養成することが出来ることが指摘された。 マスワクチネーションを行う場合の接種人員の確保には、事前の計画が必要。 入国時接種の際の善感確認は、居住区の保健所が行う。 Ⅲ具体的な予防接種実施方法	想定に基づいた計算式を提示 接種者が行うのが原則だが、検疫では確認は困難。 変更なし

⑨ 医療体制

主な変更点	変更理由
医療体制	変更なし

⑩ 治療指針

主な変更点	変更理由
Ⅰ天然痘患者の治療	変更なし
Ⅱ感染拡大の予防	変更なし

Ⅲ　シドフォビル(ビスタイド)を削除した Ⅳ抗ワクシニア免疫グロブリン(VIG)より ~~Ⅳ~~Ⅲ抗ワクシニア免疫グロブリン(VIG)に変更	シドフォビルについては、天然痘への有効性は、実験動物の段階であり、国内未承認であることからも、国の指針の第一選択に掲載することは、現時点で適切ではない。また、経口の ST246 が開発されていることから、削除が望ましい。 (参考文献) Smith SK, Olson VA, Karem KL, Jordan R, Hruby DE, Damon IK, In vitro efficacy of ST246 against smallpox and monkeypox. Antimicrob Agents Chemother. 53:1007-12, 2009

⑪ 広報及び情報提供

主な変更点	変更理由
Ⅲ 各状況レベルの対応　を変更 ・レベル Ⅲa→Ⅲb は厚労省と自治体が協議してレベル変更を決定し、自治体がレベル Ⅲb の宣言と説明を行うことを記載。	基本方針で提案するレベル分けの方針に準じるため。 レベル Ⅲa→Ⅲb の変更は自治体単位で行うことを想定しているため、発表体制に関しても明示した。

平成24年度厚生労働科学研究費補助金(健康安全・危機管理対策総合研究事業)
「バイオテロのリスク評価、数理モデルの開発とガイドラインの整備、臨時予防接種の円滑な実施できる体制についての検討」

分担研究報告書
「天然痘・肺ペスト・炭疽菌を用いたバイオテロの全国シミュレーションの開発」

国立感染症研究所感染症情報センター　大日康史
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要旨
【目的】肺ペストを用いたバイオテロの流行予測及び対策の評価のために、全国民を対象としたシミュレーションを実施できるシステムを確立する。
【方法】対策として、対策なし、全国民に対する抗生物質・ワクチン投与、濃厚接触者のみへの抗生物質・ワクチン投与、濃厚接触者の外出禁止＋抗生物質・ワクチン投与、外出自粛(自粛率20%・60%)、交通規制(長距離のみ規制・生活圏単位で規制)、学校閉鎖、職場閉鎖及びその組み合わせの内、組み合わせが可能な38種類を検討する。放出は空中散布とし、検討期間は放出後100日間、対策の実施日は30日目とする。
【結果と考察】予算不足から、高速化へのプログラム改良や、繰り返し計算のための計算能力を十分量確保できず、繰り返し計算は実施できなかった。また、危機管理部局での実際の使用に耐えるように、例えば対策実施の開始日、あるいは対策のパラメーターを危機管理部局が任意に変更できるような、改良が必要である。

A. 研究目的

従来のバイオテロ評価やパンデミックの事前評価のためのシミュレーションでは、人々の移動のデータとしてPTデータを用いてきた[1)]ことから、それが調査されている大都市部のみに限定されており、また都市間の移動も十分には考慮されてこなかった。昨年度までに、全国民を対象としたシミュレーションを実施できるシステムを確立し、バイオテロでの使用が危惧される天然痘に応用した。本年度は、それを肺ペストに応用する。

B. 材料と方法

全国民を対象としたシミュレーションとするために国勢調査での通勤通学データを用いて、全国民の移動のデータベースを作成する。また高速化は、主にアルゴリズムの見直しで行う。また、国勢調査の移動では十分にとらえられない出張等の長距離移動の情報を、「感染旅客流動の実態(2005)」から得てシミュレーションに加える等の開発を行う。

対策として、

- 対策なし
- 全国民に対する抗生物質・ワクチン投与
- 濃厚接触者のみへの抗生物質・ワクチン投与
- 濃厚接触者の外出禁止＋抗生物質・ワクチン投与

- 外出自粛(自粛率 20%)
- 外出自粛(自粛率 60%)
- 交通規制(長距離のみ規制)
- 交通規制(生活圏単位で規制)
- 学校閉鎖
- 職場閉鎖

及びその組み合わせの内、組み合わせが可能な38種類を検討する。放出は空中散布とし、検討期間は放出後 100 日間、対策の実施日は 30 日目とする。生物剤としては、肺ペストを検討する。効果の指標としては死亡者の対策なしからの削減率(救命率)を用いる。

C. 結果

生物剤と対策の組み合わせ毎に一回づつのシミュレーションを実施した結果が表1にまとめられている。

D.考察

シミュレーションモデルの開発は成功したが、本来であれば生物剤ごとに対策ごとに数百回の繰り返し計算を行い、その信頼区間をもって対策の評価を行うべきである。しかしながら予算不足から、高速化へのプログラム改良や、繰り返し計算のための計算能力を十分量確保できず、繰り返し計算は実施できなかった。今後の課題としたい。

また、危機管理部局での実際の使用に耐えるように、例えば対策実施の開始日、あるいは対策のパラメーターを危機管理部局が任意に変更できるような、改良が必要である。

E.結論

全国民を対象としたシミュレーションを確立し、その実用例を示した意義は大きい。

F. 健康危険情報

特になし

G. 論文発表

論文発表

特になし

学会等での報告

特になし

H. 知的財産権の出願・登録状況

(予定を含む)

特になし

表1　肺ペストにおける救命率（%）

政策項目	政策なし	抗生物質投与	外出自粛（自粛率20%）	外出自粛（自粛率60%）	交通規制（長距離のみ規制）	交通規制（生活圏単位で規制）	患者家族への抗生物質投与	患者家族の外出禁止＋抗生物質投与	学校閉鎖	職場閉鎖
政策なし	ベース	42.1	35.1	31.9	33.6	49.9	33.9	33.7	5.7	50.7
抗生物質投与										
外出自粛（自粛率 20%）				−	35.5	69.1	35.1	36.1	−	−
外出自粛（自粛率 60%）					32.2	69.8	32.3	32.2	−	−
交通規制（長距離のみ規制）						50.0	34.0	33.5	36.5	50.5
交通規制（生活圏単位で規制）							50.5	50.6	0.5	
患者家族への抗生物質投与								−		51.2
患者家族の外出禁止＋抗生物質投与										51.1
学校閉鎖										−
職場閉鎖										

−：組み合わせ上実施不可能

Ⅲ　研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

論文

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
石原雅之, 藤田真敬, 森　康貴, 岸本聡子, 服部秀美, 山本頼綱, 立花正一, 金谷泰宏	生物・化学剤の除染技術の動向	防医大誌	37(1)	8-17	2012
Satoko Kishimoto, Masayuki Ishihara, Shingo Nakamura, Masanori Fujita, Megumi Takikawa, Yuki Sumi, Tomoharu Kiyosawa, Toshinori Sato, Yasuhiro Kanatani	Fragmin/protamine microparticles to adsorb and protect HGF and to function as local HGF carriers in vivo	Acta Biomaterialia	9	4763-4770	2013

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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 μg 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 (⩾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

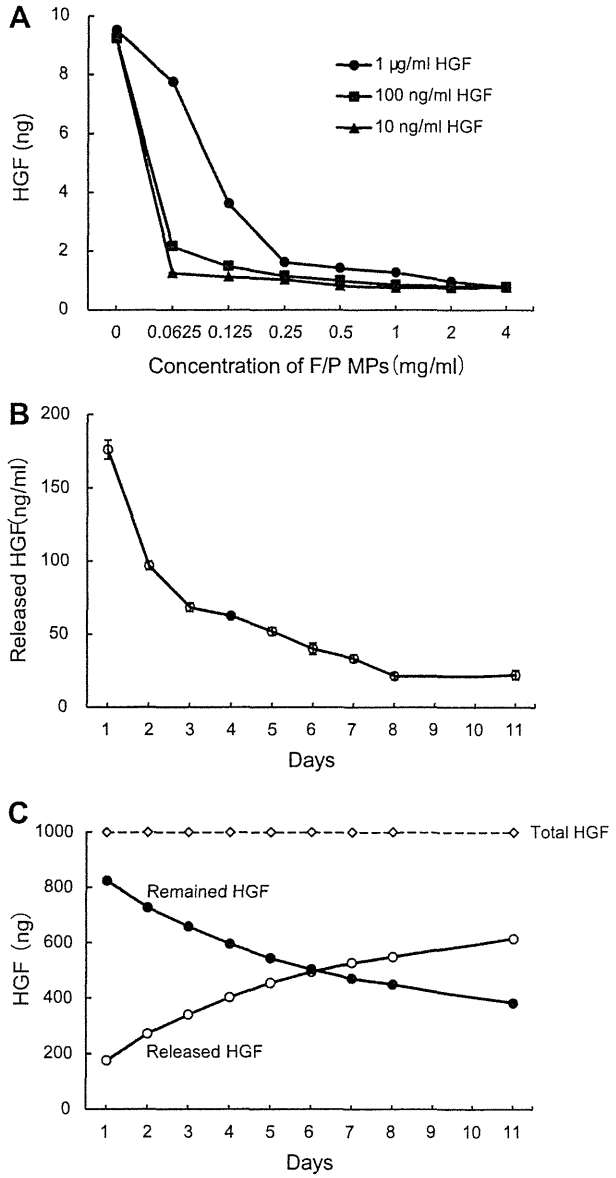

Fig. 1. (A) Absorption of HGF onto F/P MPs. The indicated amounts of F/P MPs were added to DMEM containing FBS and HGF. The solution was centrifuged to precipitate HGF-containing F/P MPs, and HGF remaining in the supernatant was measured by ELISA. (B) HGF release into DMEM. HGF release into DMEM on a 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.

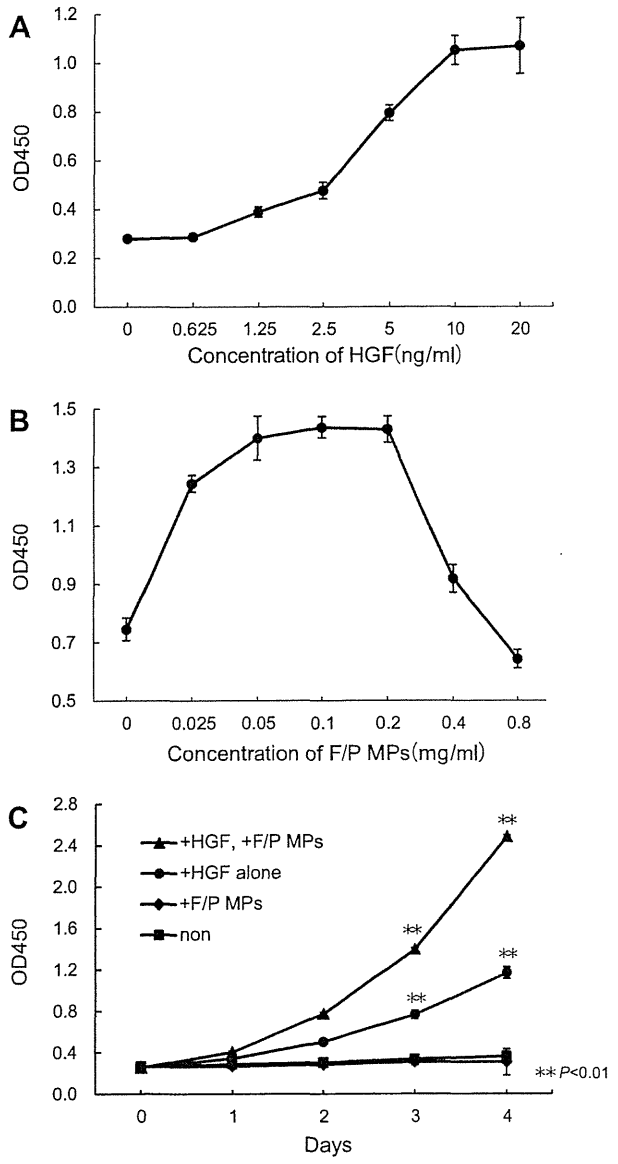

Fig. 2. (A) Influence of HGF concentration on MVEC growth in DMEM containing 5% FBS without F/P MPs. (B) Influence of F/P MP concentration on MVEC growth in DMEM containing FBS with HGF (5 ng ml^{-1}). (C) Influence of F/P MPs (0.1 mg ml^{-1}) and HGF on MVEC growth in DMEM containing FBS. Each data 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

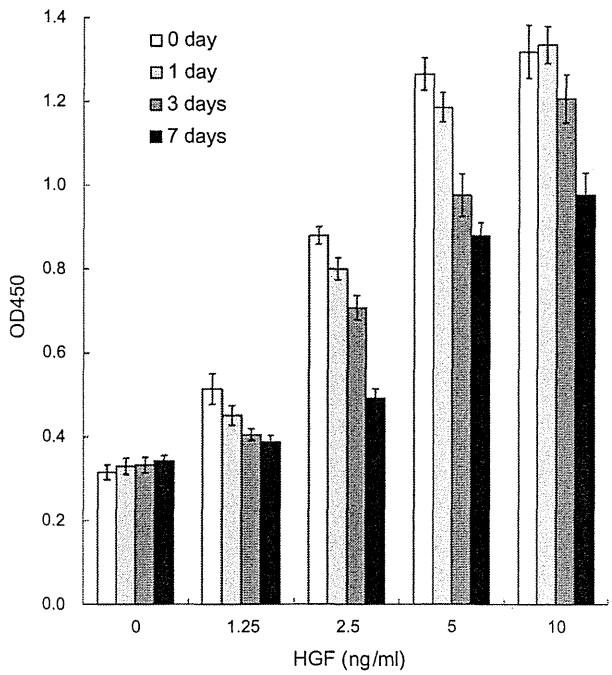

Fig. 3. The stability of pre-immobilized HGF as determined by MVEC proliferation assay. F/P MPs were incubated with HGF. After incubating the HGF-containing F/P MP stock solution for the indicated time periods MVECs were cultured in DMEM containing FBS and diluted pre-immobilized HGF-containing F/P MPs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

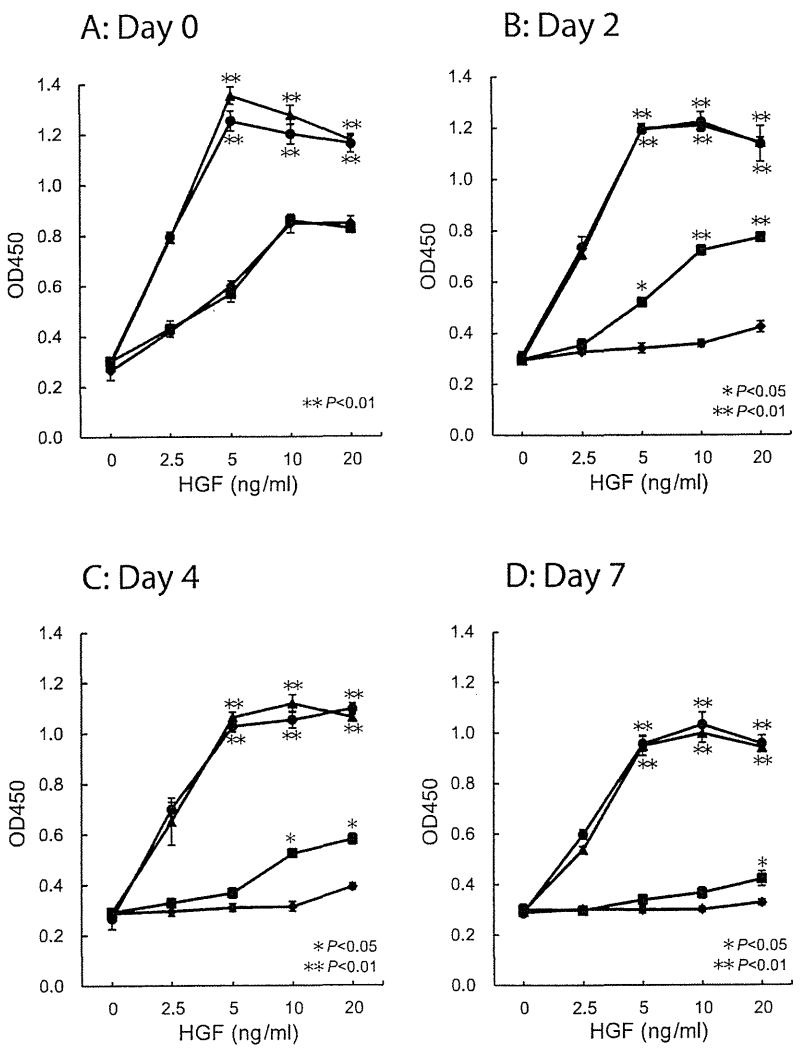

Fig. 4. The protective effect of F/P MPs on HGF. Stock solutions of HGF-containing F/P MPs with (●) or without (▲) FBS and HGF alone with (■) or without (♦) FBS were incubated for (A) 0, (B) 2, (C) 4, and (D) 7 days. Stock solutions were diluted to the indicated nominal concentrations of HGF-containing 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

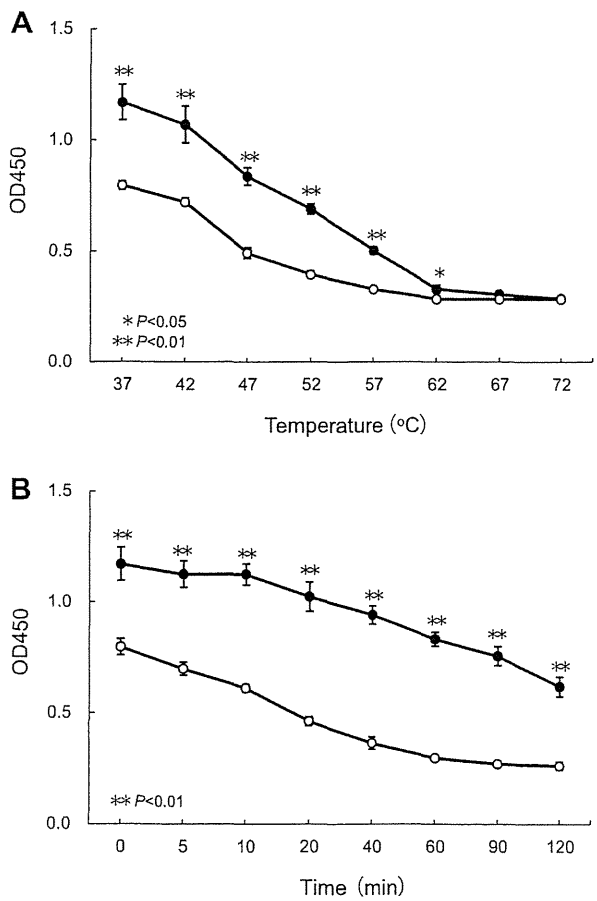

Fig. 5. The protective effect of F/P MPs on the bioactivity of (A) heat-treated and (B) trypsin-treated HGF. (A) The stock solutions (●) and HGF alone (○) were incubated at the indicated temperatures. The heated HGF solutions were diluted with DMEM containing FBS. MVECs were cultured using one of the 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

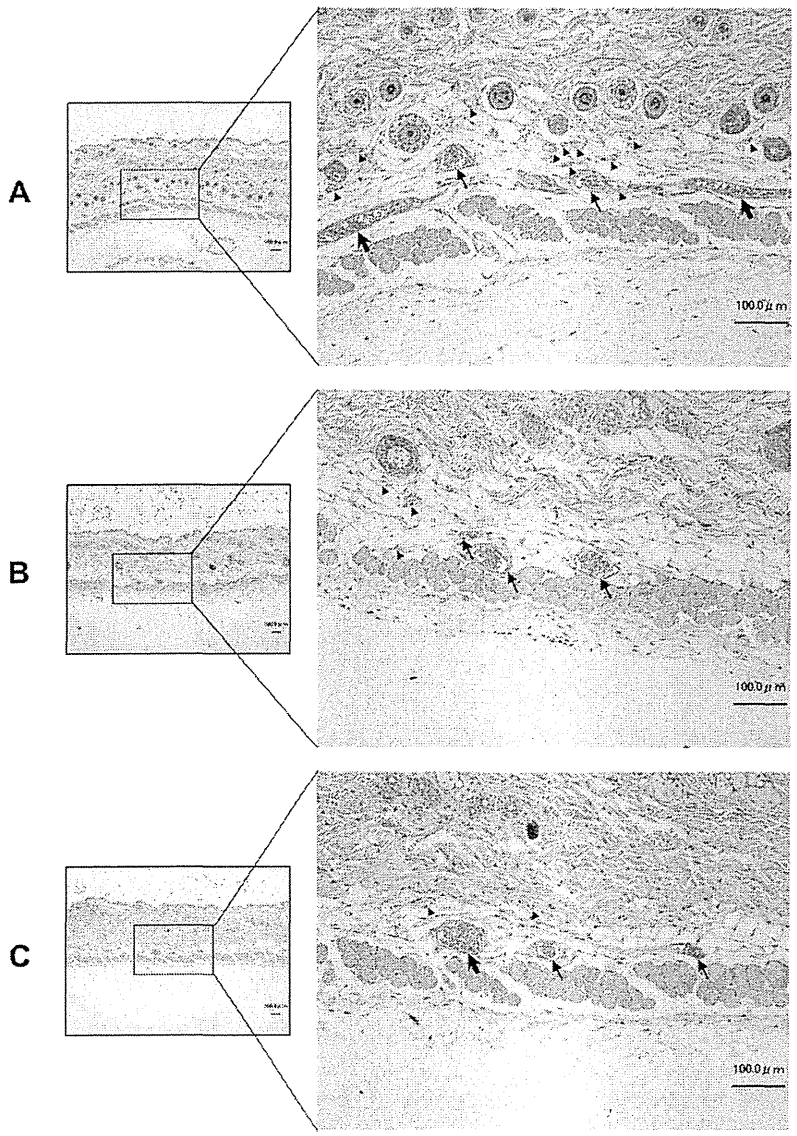

Fig. 6. Histological examination of subcutaneous sites 8 days after injection of (A) HGF-containing F/P MPs, (B) HGF alone, or (C) F/P MPs alone. Photographs are representative H&E stained tissue samples from 10 sites (1 per mouse) injected with either HGF-containing F/P MPs, HGF alone, or F/P MPs alone.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

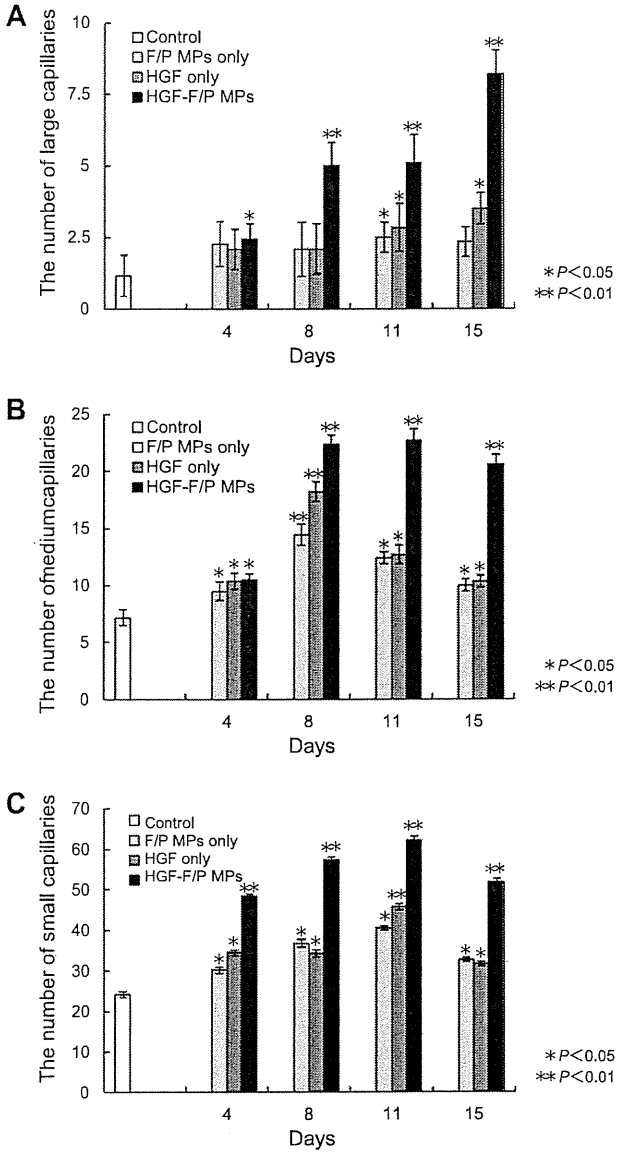

Fig. 7. Angiogenic effect of HGF-containing F/P MPs in vivo. The number of large, medium, and small capillaries in each sample (about 10 × 1.0 mm) was counted ($n = 10$). All values are means ± SD. An unpaired t-test was used to compare the experimental and control groups. $^{**}P < 0.01$, $^{*}P < 0.05$.

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.

Acknowledgements

We acknowledge the expertise and advice of Associate Prof. Koichi Fukuda and also thank the personnel of the Institute of Laboratory Animals, Graduate School of Medicine, National Defense Medical College for expert care of the animals. This study was partially supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan under Grant No. 1058500. S.K. was supported by a Restart Postdoctoral Fellowship of the Japan Society for the Promotion of Science.

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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総　説

生物・化学剤の除染技術の動向

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山本頼綱**，立花正一**，金谷泰宏****

防医大誌（2012）37（1）：8－17

要旨：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，現場の復旧のために求められる。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る必要がある。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検討した。

索引用語：　生物化学剤の除染　／　銀ナノ粒子／キトサン複合体　／　吸着性材料　／　酸化チタンナノ粒子　／　光触媒技術

はじめに

1995年の東京地下鉄サリン事件や2001年のアメリカ合衆国郵便物炭疽菌事件において大量破壊兵器である化学兵器用剤（Chemical warefare agent；化学剤）や生物兵器用剤（Biological warefare agent；生物剤）が使用され，一般市民に多大な被害（サリン事件；12名死亡，約5000名傷害，炭疽菌事件；5名死亡，十数名傷害）が引き起こされ，強烈なテロの脅威を与えた。このように生物化学剤などの危険物を用いたテロリズムの脅威は顕在化しており，国家的な取り組みが求められているところである[1]。生物化学テロの危機管理のために医学研究者は，いかに貢献すべきか問われているが，専門性を考慮すれば，生物化学剤の除染，防護，解毒，治療に取り組むべきであると考えられる。本総説では，まず対象となる生物化学剤の種類・性状について述べ，次に除染のニーズとコンセプトについて解説する。そして除染の現状技術について解説し，最後に最近の除染剤の研究・開発の動向について，報告者らが実施している生物化学剤の除染剤の研究・開発を含めて紹介する。

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生物化学剤の特徴

1．化学剤の特徴

化学剤は低分子の合成化合物であり，その作用により，神経剤，びらん剤，血液剤（シアン化化合物），窒息剤等の分類される（表1）。物性的にも，一般的に常温・常圧で気体である化学剤，揮発性の液体である化学剤等様々である。化学テロにおいては，蒸気又はエアロゾルとして散布され，曝露した被害者が吸入などで体内に摂取して毒性を受けるので，毒性値として吸入半数致死量（LC_{50}；mg-min/m^3）を示している[2]。

（1）神経剤

神経剤は，神経シナプスのコリンエステラーゼ活性を阻害し，アセチルコリンを蓄積させ，神経伝達をかく乱する作用により毒性を発揮する[3]。化学剤のなかでは即効性であり，最も毒性が高い。通常無色，無臭で五感による検知は困難である。

（2）びらん剤

びらん剤は，目と呼吸器を侵し，皮膚を糜爛する。代表的なものにマスタードとルイサイトが知られているが，水中で反応性の高いアルキル化剤として作用し，生体高分子（たんぱく質や核酸等）をアルキル化する。特有の臭気により五感での検知は比較的容易である。これらマスタードとルイサイトは第一次世界大戦後半から使われ始め，現在もなお多くの国で備蓄されていると言われている。旧日本軍も大量に製造しており，びらん剤弾薬を中国満州地区や国内に遺棄し，現在はその遺棄化学剤処理が日本政府の責務となっている[4]。

（3）血液剤

血液剤は，呼吸により身体に吸収され，循環器及び呼吸器を侵し，昏睡と全身痙攣を引き起こす。特有の臭気により五感での検知は容易であり，持久効果は殆どない。

（4）窒息剤

窒息剤は，鼻，喉，気管，肺等の呼吸器に障害を与え，呼吸困難を引き起こすことにより毒性を発揮する。特有の臭気により五感での検知は容易であり，持久効果は殆どない。

2．生物剤の特徴

生物剤については，ここでは化学剤との違いについて解説する。米国集団疾病予防管理センター（CDC：Center for Disease Control and Prevention）は，攻撃対処準備上の優先順位が高い生物剤を①ヒト—ヒト感染の可能性が大きいもの，②死亡率が高く公衆衛生に与える影響

表1．代表的な有毒化学剤の種類・性状

分類	名　称	記号	臭　い	作用速度	持　久　性（常　温）	半数致死量（mg-min/m^3）
神経剤	タブン	GA	無　臭	極めて速い	1～2日	400（吸入）
	サリン	GB	無　臭		水とほぼ同じ	100（吸入）
	ソマン	GD	果実臭		1～2日	100（吸入）
	V剤	VX	無　臭	速　い	3日～3週	10（吸入）
びらん剤	マスタード	HD	にんにく臭	遅　い（数時間以上）	2日～7日	1,500（吸入）
		HN	魚臭又はかび臭			
	ルイサイト	L	ゼラニュム臭	速　い	HDより短い	1,300（吸入）
血液剤	青　酸	AC	アーモンド臭	極めて速い	2～3分	2,600（吸入）
	塩化シアン	CK		速　い		
窒息剤	ホスゲン	CG	新しい乾草	速　い	2～3分	3,200（吸入）
	ジホスゲン	DP	トウモロコシ臭			
備考	・20℃における剤の状態は，ほとんど液体である ・半数致死量とは，無防護の人員の50%が死亡する化学剤の量で，空気1 m^3中に含まれる化学剤の量（mg）に暴露した時間（分）を乗じたもの					

持久性：局所毒性の半減期　　文献2，3から引用，改変。

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

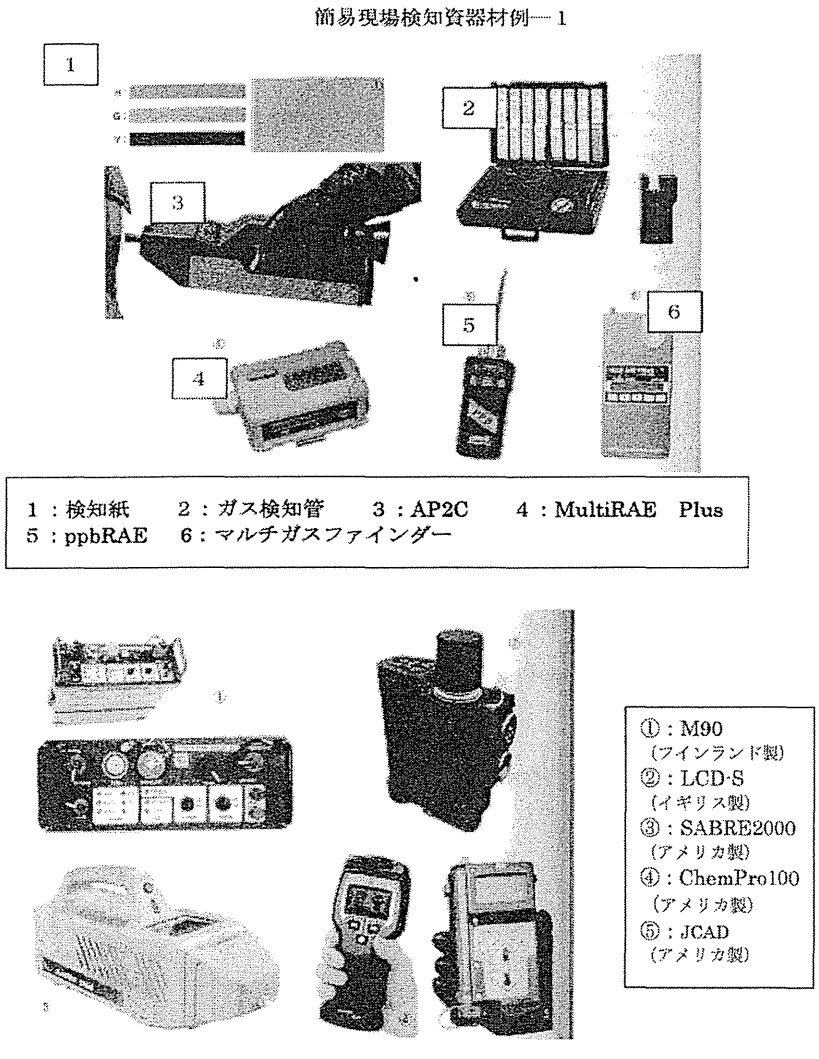

図1．簡易現場検知資器材（可搬・汎用性）
文献7より引用，改変。