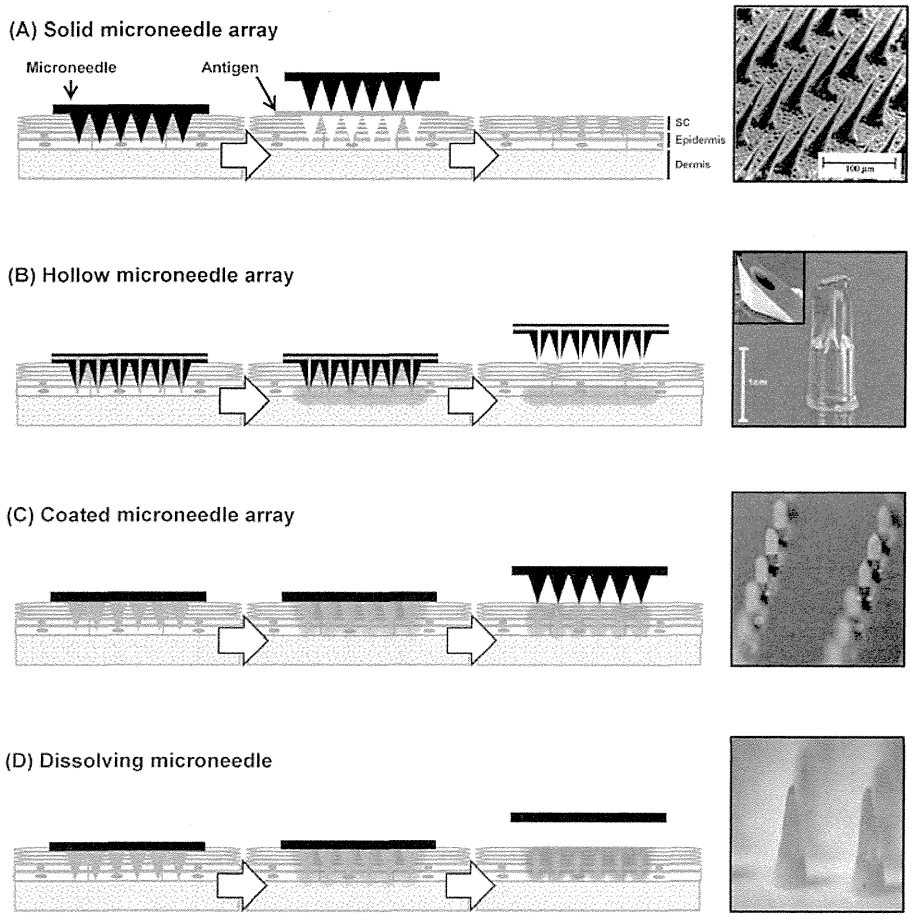

Fig. 2. Examples of Transcutaneous Delivery Using Microneedle Array

A: Deliver antigen into the skin through the puncture holes created using a microneedle array, B: Inject antigen into the skin through a hollow microneedle array, C: Diffuse antigen into the skin using an antigen-coated microneedle array; D: Deliver antigen into the skin with dissolution of the antigen-coated or antigen-loaded microneedle array.

ない．そこで開発されたのが，ソリッドマイクロニードルの表面にワクチンをコーティングし，その微小針を皮膚に穿刺することでワクチン抗原を皮膚内へ拡散させる手法である［Fig. 2(C)］.[31] 本手法では，マイクロニードル表面にワクチンが乾燥状態で吸着しているため，溶液状態における保管よりもワクチンの安定性が高いと考えられており，生きた細菌やウイルスを使用する生ワクチンへの応用も図られている.[32] 現在，マイクロニードルの素材やコーティング溶液の組成を最適化することで，ワクチン抗原をより多く，より安定に保持できるコーティングマイクロニードル製剤の開発が進められている．

4-4. 溶解型マイクロニードル　上述した3種類のマイクロニードルは第一世代マイクロニードルに分類され，シリコンや金属(ステンレス，チタン)を材料として作製されている．第一世代マイクロニードルは剛性に優れる，成形し易い，といった利点を有している一方で，生体内で折れ残り，重篤な組織傷害を引き起こす危険性を払拭できないことが実用化を困難にしている．そこで近年では，第二世代マイクロニードルとして，生分解性バイオポリマーや生体由来成分であるヒアルロン酸，コンドロイチン硫酸などを利用した溶解型マイクロニードルの開発が注目されている［Fig. 2(D)］.[33-35] 第二世代マイクロニードルは，生体適合性に優れる素材を使用し，マイクロニードル自体が溶解することによって装填あるいは吸着したワクチン抗原を皮膚内へと送達するといった特徴を有する．そのため，第一世代マイクロニードルの安全面における問題点を克服できると考えられており，臨床応用・実用化が期待されている．

5. 皮膚内溶解型マイクロニードルパッチを用いた経皮ワクチン製剤

筆者らはコスメディ製薬株式会社との共同研究により独自に開発した皮膚内溶解型マイクロニードルパッチ（MicroHyala®; MH）を用いた経皮ワクチン製剤の開発を進めている.[35-38] MH は皮膚組織成分であるヒアルロン酸を主成分としており，マイクロニードルの形状や長さは自由に調整することができる［Fig. 3(A)］. MH をマウスやラットの背部皮膚に貼付すると，30 分以上の貼付によって針は完全に溶解し，その針部に装填できる物質であれば可溶性タンパク質のみならず，粒子状物質をも生きた表皮並びに真皮へと確実に送達可能である［Fig. 3(B)］.[35] そこで，可溶性タンパク質抗原である破傷風・ジフテリアトキソイドを装填した MH 製剤をラットの背部皮膚に貼付したところ，従来の注射ワクチン接種群と同様のプロファイルで血中トキソイド特異的抗体価が上昇し，それらの抗体は毒素に対する中和活性を発揮した.[36] また，粒子状抗原であるインフルエンザ HA 抗原を装填した MH 製剤をマウス背部皮膚に貼付した際にも筋肉内注射接種（intramuscular injection; IMI）群に匹敵する HA 特異的抗体産生が認められ［Fig. 4(A)］，産生された抗体のインフルエンザウイルス感染阻止活性（HA 抗原の赤血球凝集に対する阻止活性；HI 価）については，経皮接種（transcutaneous immuniza-

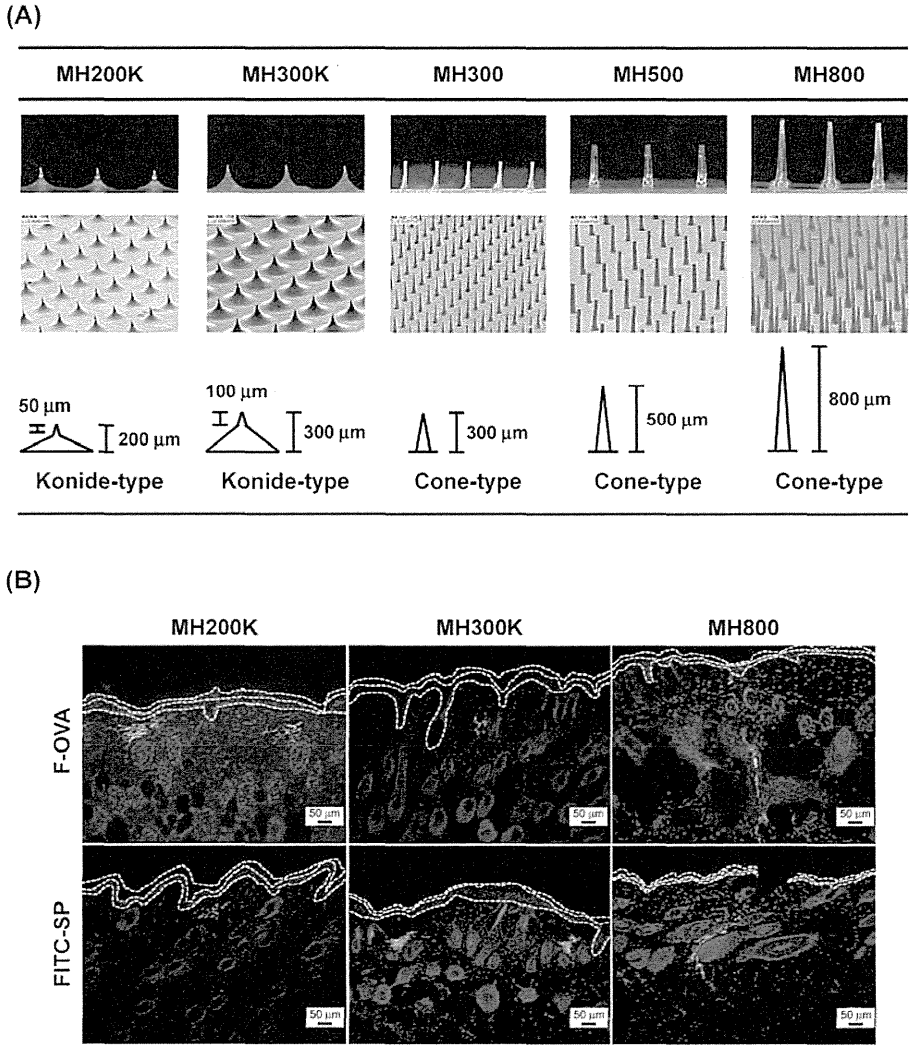

Fig. 3. Dimension (A) and Antigen Delivery Properties (B) of Self-dissolving Microneedle Array (MicroHyala; MH)

A: The microneedle of the MH series is controlable to various shapes and length. B: MH200K, MH300K, or MH800 loaded with fluorescein-labeled ovalbumin (F-OVA) or fluorescein isothiocyanate-labeled silica particles (300 nm in diameter; FITC-SP) were applied on the back skin of mice for 1 h. The skin frozen section (8-μm thick) were photographed under a fluorescence microscope. The nucleus was counterstained using 4′,6-diamidino-2-phenylindole (DAPI). The white dotted lines indicate the surfaces of the stratum corneum, epidermis, and superficial dermis, respectively, from top to bottom.

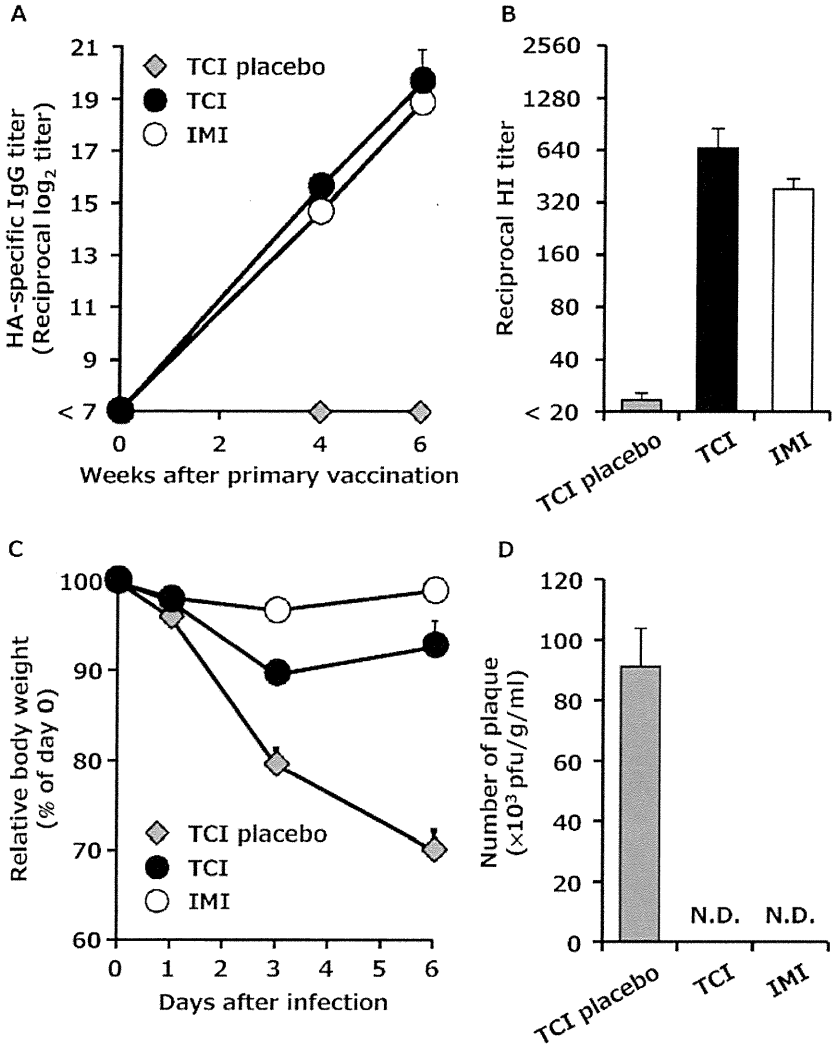

Fig. 4. Protection of Vaccinated Mice against Influenza Virus Challenge

BALB/c mice were transcutaneously vaccinated with MH containing 0.4 μg of hemagglutinin (HA) antigen from influenza virus [A/PR/8/34(H1N1)] for 6 h twice at 4-week intervals (TCI group). Control groups were transcutaneously treated with MH application without HA antigen (TCI placebo group) or intramuscularly injected with 0.4 μg of HA antigen (IMI group), twice at 4-week intervals. These mice were intranasally infected with 6×10^5 plaque-forming unit (PFU) of the A/PR/8/34 (H1N1) virus. A: At the indicated points, sera collected from these mice were assayed for the HA-specific IgG titer by ELISA. B: Two weeks after the final vaccination, sera collected from these mice were assayed for the hemagglutination inhibition (HI) titer. C: Body weight was measured and is presented as a percentage of the initial weight before infection (day 0). D: Six days after infection, the lungs were collected from these mice and number of viruses in the lung homogenate was determined using a plaque assay system. Data are expressed as mean±S.E. of results from 13 (A and B) or 10 (C and D) mice.

tion; TCI) 群のほうが IMI 群よりも高値を示した [Fig. 4(B)].[36] さらに，これらワクチン投与マウスにインフルエンザウイルスを経鼻感染させたところ，プラセボ群では著しい体重減少や肺組織内でのウイルス増殖が認められたのに対し，TCI 群では全例においてそれらの感染症状を示さず，MH 製剤が感染防御に非常に効果的な経皮ワクチン製剤であることが実証された [Figs. 4(C) and (D)].[36]

これらの基礎・前臨床研究の成果に基づき，筆者らは MH を応用したインフルエンザ経皮ワクチン製剤のヒトでの安全性及び有効性を検証すべく，倫理委員会の審査・承認の下健康成人男性ボランティアを対象とした臨床研究を実施した．まずパイロットスタディーとして，針長 800 μm の MH をヒト皮膚に適用した際の針部溶解性を観察したところ，貼付 6 時間後には対象とした 3 人の被験者全員において MH の針部は根元まで溶解した [Fig. 5(A)].[38] また，MH 貼付後の皮膚を共焦点レーザー生体顕微鏡により観察すると，皮膚表面には整列した微小針による穿刺孔が多数認められ，三次元画像の構築からそれらの穿刺孔が確実に角質層を突破して生きた表皮・真皮にまで到達していることを確認した

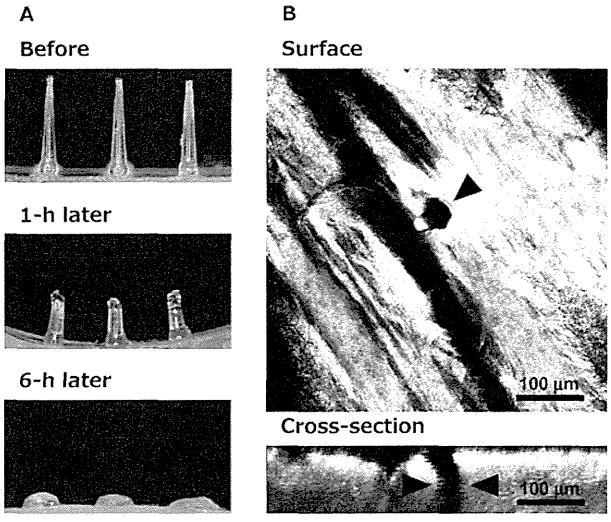

Fig. 5. Needle-dissolution of MH800 (A) and Status of Skin Treated with MH800 (B) in Humans

The MH800 was applied to the skin of left lateral upper arm of healthy volunteers. A: One or 6 h later, the microneedle arrays were observed under stereoscopic microscope. B: Five seconds after MH800-application, skin images were photographed under *in vivo* confocal scanning laser microscope.

[Fig. 5 (B)].[38] そこで，20 人の被験者を対象に MH を 6 時間貼付した後の経過観察・問診・血液検査を実施したところ，重篤な皮膚局所反応や全身性の副作用は認められず，MH がヒト皮膚に対しても安全に適用できる経皮ワクチンデバイスであることが示された.[38] これらの結果を踏まえて，三価季節性インフルエンザ HA 抗原を装填した MH 製剤を作製し，20 人の被験者に 3 週間隔で 2 回貼付した際の安全性及び有効性を従来の皮下注射接種した被験者群と比較する臨床研究プロトコールを実施した．HA 抗原装填 MH 製剤を貼付した被験者において，重篤な皮膚刺激性・起炎性は認められず，全身性の副反応を伴うことなく皮下注射群とほぼ同等の血中 HI 抗体価の上昇が確認できた（投稿準備中）．本臨床研究結果は，筆者らの MH を応用した経皮ワクチン製剤が注射に代わる簡便，低侵襲，安全，有効なワクチン接種法として極めて有望であることを示すものであり，新規剤形ワクチンとして実用化されれば，乳幼児のワクチン接種の負担を大きく軽減できるのみならず，感染症地域への渡航者に対する事前予防ワクチンの接種，開発途上国へのワクチン普及など，世界的な感染症対策に大きく貢献できるものと考える．

6. おわりに

従来の注射ワクチン製剤に代わる新規剤形ワクチンとして，「貼るワクチン」のほかにも抗原を経鼻投与する「吸うワクチン」[39,40]や経口投与する「飲むワクチン」[41,42]の開発が進められており，最近では経鼻噴霧型のインフルエンザ生ワクチンとして FluMist® (MedImmune) が米国で承認・実用化された．わが国においてもこのような次世代型ワクチンの研究に多大な国家予算が投じられており，新規ワクチンに対する非臨床・臨床・アジュバントのガイドラインの策定など，産学官連携体制によってその開発を加速する対策もとられている．革新的ワクチン技術に対する社会的ニーズの高まりに対して，筆者ら独自の MH を応用した経皮ワクチン製剤は，ワクチン接種を簡便，安全，安価にすることでワクチンの世界的普及を強力に推進し，感染症に対して安全・安心な社会の実現に大きく貢献できる可能性を有する．本稿で紹介した基礎研究から臨床研究までの成果が，近い将来，世界初，日本発の理想的なワクチン製剤の上市という形で予防医療の一翼を担うことを期待したい．

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REFERENCES

1) Valladeau J., Saeland S., *Semin. Immunol.*, **17**, 273–283 (2005).

2) Sugita K., Kabashima K., Atarashi K., Shimauchi T., Kobayashi M., Tokura Y., *Clin. Exp. Immunol.*, **147**, 176–183 (2007).
3) Barry B. W., *Nat. Biotechnol.*, **22**, 165–167 (2004).
4) Belshe R. B., Newman F. K., Cannon J., Duane C., Treanor J., Van Hoecke C., Howe B. J., Dubin G., *N. Engl. J. Med.*, **351**, 2286–2294 (2004).
5) Kenney R. T., Frech S. A., Muenz L. R., Villar C. P., Glenn G. M., *N. Engl. J. Med.*, **351**, 2295–2301 (2004).
6) Laurent P. E., Bonnet S., Alchas P., Regolini P., Mikszta J. A., Pettis R., Harvey N. G., *Vaccine*, **25**, 8833–8842 (2007).
7) Prymula R., Usluer G., Altinel S., Sichova R., Weber F., *Adv. Ther.*, **29**, 41–52 (2012).
8) Weaver J. C., *Methods Mol. Biol.*, **55**, 3–28 (1995).
9) Zhao Y. L., Murthy S. N., Manjili M. H., Guan L. J., Sen A., Hui S. W., *Vaccine*, **24**, 1282–1290 (2006).
10) Cristillo A. D., Weiss D., Hudacik L., Restrepo S., Galmin L., Suschak J., Draghia-Akli R., Markham P., Pal R., *Biochem. Biophys. Res. Commun.*, **366**, 29–35 (2008).
11) Hirao L. A., Wu L., Khan A. S., Satishchandran A., Draghia-Akli R., Weiner D. B., *Vaccine*, **26**, 440–448 (2008).
12) Kelly K., Loskutov A., Zehrung D., Puaa K., LaBarre P., Muller N., Guiqiang W., Ding H. G., Hu D., Blackwelder W. C., *Vaccine*, **26**, 1344–1352 (2008).
13) Roberts L. K., Barr L. J., Fuller D. H., McMahon C. W., Leese P. T., Jones S., *Vaccine*, **23**, 4867–4878 (2005).
14) Jones S., Evans K., McElwaine-Johnn H., Sharpe M., Oxford J., Lambkin-Williams R., Mant T., Nolan A., Zambon M., Ellis J., Beadle J., Loudon P. T., *Vaccine*, **27**, 2506–2512 (2009).
15) Aboud S., Nilsson C., Karlén K., Marovich M., Wahren B., Sandström E., Gaines H., Biberfeld G., Godoy-Ramirez K., *Clin. Vaccine Immunol.*, **17**, 1124–1131 (2010).
16) Gupta P. N., Mishra V., Rawat A., Dubey P., Mahor S., Jain S., Chatterji D. P., Vyas S. P., *Int. J. Pharm.*, **293**, 73–82 (2005).
17) Schlosser E., Mueller M., Fischer S., Basta S., Busch D. H., Gander B., Groettrup M., *Vaccine*, **26**, 1626–1637 (2008).
18) Glenn G. M., Taylor D. N., Li X., Frankel S., Montemarano A., Alving C. R., *Nat. Med.*, **6**, 1403–1406 (2000).
19) Naito S., Maeyama J., Mizukami T., Takahashi M., Hamaguchi I., Yamaguchi K., *Vaccine*, **25**, 8762–8770 (2007).
20) Ishii Y., Nakae T., Sakamoto F., Matsuo K., Matsuo K., Quan Y. S., Kamiyama F., Fujita T., Yamamoto A., Nakagawa S., Okada N., *J. Control. Release*, **131**, 113–120 (2008).
21) Matsuo K., Ishii Y., Quan Y. S., Kamiyama F., Mukai Y., Yoshioka Y., Okada N., Nakagawa S., *J. Control. Release*, **149**, 15–20 (2011).
22) Matsuo K., Ishii Y., Quan Y. S., Kamiyama F., Mukai Y., Okada N., Nakagawa S., *Biol. Pharm. Bull.*, **34**, 586–589 (2011).
23) Matsuo K., Ishii Y., Quan Y. S., Kamiyama F., Asada H., Mukai Y., Okada N., Nakagawa S., *Biol. Pharm. Bull.*, **34**, 1835–1840 (2011).
24) Hirobe S., Matsuo K., Quan Y. S., Kamiyama F., Morito H., Asada H., Takaya Y., Mukai Y., Okada N., Nakagawa S., *Vaccine*, **30**, 1847–1854 (2012).
25) Matsuo K., Ishii Y., Kawai Y., Saiba Y., Quan Y. S., Kamiyama F., Hirobe S., Okada N., Nakagawa S., *J. Pharm. Sci.*, **102**, 1936–1947 (2013).
26) Gerstel M. S., Place V. A., U.S. Patent No. 3, 964, 482 (1976).
27) Henry S., McAllister D. V., Allen M. G., Prausnitz M. R., *J. Pharm. Sci.*, **87**, 922–925 (1998).
28) Ding Z., Verbaan F. J., Bivas-Benita M., Bungener L., Huckriede A., van den Berg D. J., Kersten G., Bouwstra J. A., *J. Control. Release*, **136**, 71–78 (2009).
29) Mikszta J. A., Alarcon J. B., Brittingham J. M., Sutter D. E., Pettis R. J., Harvey N. G., *Nat. Med.*, **8**, 415–419 (2002).
30) Van Damme P., Oosterhuis-Kafeja F., Van der Wielen M., Almagor Y., Sharon O., Levin Y., *Vaccine*, **27**, 454–459 (2009).
31) Gill H. S., Prausnitz M. R., *Pharm. Res.*, **24**, 1369–1380 (2007).
32) Hiraishi Y., Nandakumar S., Choi S. O., Lee

J. W., Kim Y. C., Posey J. E., Sable S. B., Prausnitz M. R., *Vaccine*, **29**, 2626–2636 (2011).

33) Sullivan S. P., Koutsonanos D. G., Del Pilar Martin M., Lee J. W., Zarnitsyn V., Choi S. O., Murthy N., Compans R. W., Skountzou I., Prausnitz M. R., *Nat. Med.*, **16**, 915–920 (2010).

34) Naito S., Ito Y., Kiyohara T., Kataoka M., Ochiai M., Takada K., *Vaccine*, **30**, 1191–1197 (2012).

35) Matsuo K., Yokota Y., Zhai Y., Quan Y. S., Kamiyama F., Mukai Y., Okada N., Nakagawa S., *J. Control. Release*, **161**, 10–17 (2012).

36) Matsuo K., Hirobe S., Yokota Y., Ayabe Y., Seto M., Quan Y. S., Kamiyama F., Tougan T., Horii T., Mukai Y., Okada N., Nakagawa S., *J. Control. Release*, **160**, 495–501 (2012).

37) Hiraishi Y., Nakagawa T., Quan Y. S., Kamiyama F., Hirobe S., Okada N., Nakagawa S., *Int. J. Pharm.*, **441**, 570–579 (2013).

38) Hirobe S., Azukizawa H., Matsuo K., Zhai Y., Quan Y. S., Kamiyama F., Suzuki H., Katayama I., Okada N., Nakagawa S., *Pharm. Res.*, **30**, 2664–2674 (2013).

39) Nochi T., Yuki Y., Takahashi H., Sawada S., Mejima M., Kohda T., Harada N., Kong I. G., Sato A., Kataoka N., Tokuhara D., Kurokawa S., Takahashi Y., Tsukada H., Kozaki S., Akiyoshi K., Kiyono H., *Nat Mater.*, **9**, 572–578 (2010).

40) van Riet E., Ainai A., Suzuki T., Hasegawa H., *Vaccine*, **30**, 5893–5900 (2012).

41) Nochi T., Takagi H., Yuki Y., Yang L., Masumura T., Mejima M., Nakanishi U., Matsumura A., Uozumi A., Hiroi T., Morita S., Tanaka K., Takaiwa F., Kiyono H., *Proc. Natl. Acad. Sci. USA*, **104**, 10986–10991 (2007).

42) Wilkhu J., McNeil S. E., Kirby D. J., Perrie Y., *Ther. Deliv.*, **2**, 1141–1164 (2011).

マイクロニードル型経皮ワクチン製剤の実用化を目指して

＊大阪大学大学院薬学研究科
薬剤学分野
廣部祥子，岡田直貴，
中川晋作

はじめに

感染症は古くから人類へ多大なる影響を与えてきた疾患であり，未だに世界における死亡原因の約4分の1を占める．また，現在の発達した交通網は国境を越えた病原体の移動を容易にしており，新興あるいは再興感染症が発生した際に瞬く間に世界的流行へと発展することが危惧されている．このような社会背景の下，感染症の阻止に最も効果的な対策となるワクチンが再び注目されている．

しかしながら，現行の注射型ワクチン製剤は投与に医療従事者を必要とするだけでなく，ワクチン製剤の輸送・保管に一貫した低温温度管理の整備が求められているため，実際にワクチンを最も必要としている開発途上国などの地域にワクチンが浸透しにくく，また感染症パンデミックやバイオテロリズム発生時にワクチンの大規模投与を迅速に施行できないという課題を有する．したがって，注射に代わる簡便で有効かつ安全な新規ワクチン手法の確立がさまざまな感染症ワクチンの有用性を向上させると考えられることから，筆者らは皮膚をターゲットとした経皮ワクチン製剤，すなわち「貼るワクチン」[1,2]の開発を進めており，その実用化に向けた取り組みを紹介する．

＊〒565-0871 大阪府吹田市山田丘1－6
E-mail sachi-be@phs.osaka-u.ac.jp

1．ワクチンの投与部位としての皮膚

皮膚は外側から，角質層，生きた表皮，真皮という順番で大きく3層に分けられる(図1)．そして皮膚は，常に外界からの異物侵入の危険にさらされているため，最外層に存在する角質層が物質透過を制限する物理的バリアーとして機能している．さらに，皮膚には生体を守るべく発達した免疫担当細胞が豊富に存在し，「免疫学的バリアー」が形成されている．ケラチノサイトは生きた表皮の約90％を占める細胞であり，各種サイトカインやケモカイン，増殖因子を分泌することで異物侵入に対する免疫反応に関与するとされている．また，抗原提示細胞(APC)として，生きた表皮にはランゲルハンス細胞(LC)が，真皮には真皮樹状細胞(dDC)が存在する．したがって，角質層下のLCやdDCに抗原を効率よく送達することができれば，抗原特異的な免疫応答を誘導できるものと考えられる．しかしながら，前述したように，皮膚には物質透過のバリアーとなる角質層が存在するため，ペプチドや蛋白質といった高分子の抗原を単に皮膚表面に塗布するだけでは，皮膚内に常在するAPCに抗原を効率よく送達することはできない．そこで筆者らは，微小な針により角質層に孔をあけることで物質を皮膚内へと送達するマイクロニードル法を用いた経皮ワクチンの開発を推進している．

2．マイクロニードルを用いた経皮ワクチンデリバリー

マイクロニードルは神経終末が存在する真皮の深部にまで針が到達しないことから，痛みを伴わずに抗原を投与することができる．マイクロニードルという概念は1976年にGerstelとPlaceらによって初めて報告され[3]，製造技術の困難さにより開発研究が停滞していたものの，近年の微細加工技術の発展に伴い，現在ではさまざまなマイクロニードルが開発されている．第一世代のマイクロニードル(図1左)[4]は，シリコンや金属(ステンレス，チタン)を構成材料としたものであり，剛性に優れる，成形しやすい，といった利点を有している[5,6]．しかし，第一世代マイクロニードルは微小針が生体内で折れ残り，重篤な組織傷害を引き起こす危険性が払拭できないために，実用化するうえで大きな課題を抱えている．そこで，第二世代マイクロニードルとして，ポリ乳酸(PLA)，ポリグリコール酸(PGA)，ポリ乳酸・グリコール酸(PLGA)といった生分解性バイオポリマーを用いることで，たとえ微小針が皮膚内で折損したとしても針自身が分解し，残存することがないマイクロ

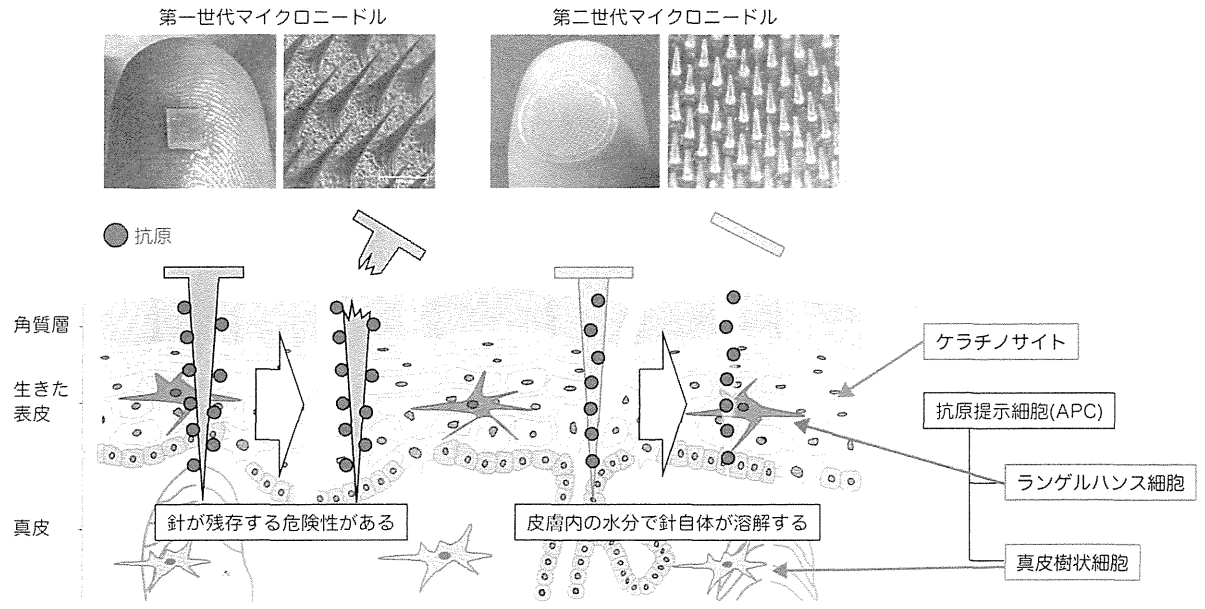

図1　マイクロニードルを用いた経皮ワクチン
皮膚は免疫応答誘導の要となる抗原提示細胞としてランゲルハンス細胞や真皮樹状細胞を有し，ワクチンの標的として優れた組織である．マイクロニードル法は，ただ貼るだけで痛みなく，抗原を皮膚内へと直接送達できる手法である．シリコンや金属で作製された第一世代マイクロニードルには皮膚内で折れて針が残存する危険性があるが，第二世代マイクロニードルは針自身が皮膚内で溶解するため安全性が高く，実用化が期待できる．写真は文献1(第二世代)，4(第一世代)より転載．

ニードルが設計された．さらに，皮膚内への薬物送達効率ならびに薬物送達速度の向上を目的に，生体成分であるヒアルロン酸やコンドロイチン硫酸ナトリウムを利用した溶解型マイクロニードルが開発された[7,8]（図1右）．これらは生体適合性に優れる構成素材を使用し，特に溶解型マイクロニードルについては，微小針自体が溶解することによって装填あるいは吸着した抗原を皮膚内へと送達することができるといった特徴を有する．このように第二世代マイクロニードルは投与後に針が消失することから，第一世代マイクロニードルが抱える安全面の問題を克服できると考えられ，臨床応用・実用化が期待される．

3．皮膚内溶解型マイクロニードルを用いた経皮ワクチンの開発

筆者らはコスメディ製薬株式会社との共同研究により，独自の皮膚内溶解型マイクロニードルを用いた経皮ワクチン製剤の開発に成功している[8〜10]．この第二世代マイクロニードルは皮膚組織成分であるヒアルロン酸を主成分としており，皮膚内の水分で針自身が溶解することで，装填物質をAPCが存在する生きた表皮ならびに真皮へと送達できる（図2A）．また動物実験において，破傷風/ジフテリアトキソイドワクチンやインフルエンザワクチンとして有効かつ安全であることを明らかとしている（図2B）．これらの研究結果をもとに，筆者らはすでに皮膚内溶解型マイクロニードルをヒト皮膚に適用する臨床研究を実施している．ヒト皮膚に針長800μmの皮膚内溶解型マイクロニードルを貼付したところ，1時間後には針の約半分が，6時間後には針全体が溶解した（図2C）．また，皮膚内溶解型マイクロニードル貼付後の皮膚を共焦点レーザー生体顕微鏡により観察した結果，皮膚表面に微小針による穿刺孔が確認され，その穿刺孔は三次元画像を構築し，皮膚断面を観察すると少なくとも深さ100μm以上にまで到達していることが判明した（図2D）．さらに，20人の健康成人被験者において，マイクロニードルの貼付による重篤な皮膚局所反応や全身性の副作用がないことを確認している[11]．このように，ヒトにおいても筆者らの皮膚内溶解型マイクロニードルは装填した物質を角質層下へと安全に送達できるこ

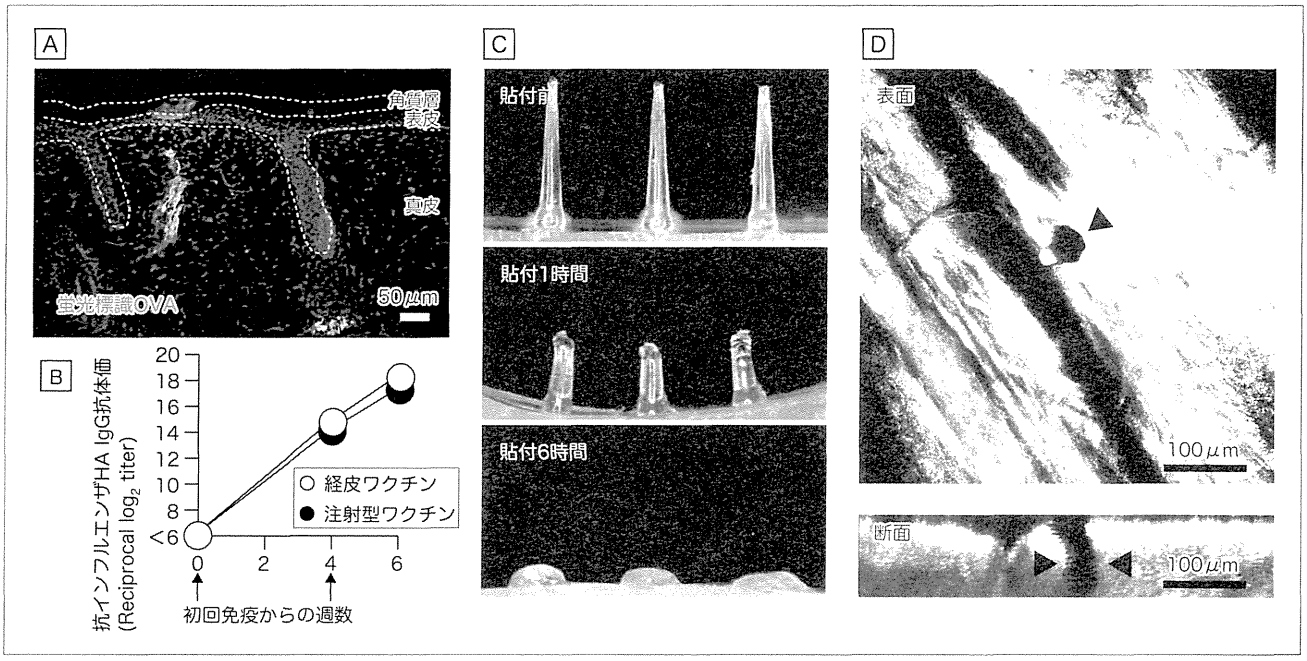

図2　皮膚内溶解型マイクロニードルによる経皮デリバリー

A 針長800μmの皮膚内溶解型マイクロニードルに蛍光標識OVAを装填し，マウス背部皮膚に6時間貼付した．貼付部位の皮膚を回収し，凍結切片を作製後，蛍光顕微鏡により観察した．文献8より転載．

B インフルエンザHA抗原〔A/Brisbane/59/2007(H1N1)〕を0.2μg装填した針長800μmのマイクロニードルパッチをマウス背部皮膚に6時間貼付した．対照群のマウスには同量のインフルエンザHA抗原を大腿部筋肉内注射した．これらのワクチン投与を4週間隔で2回実施し(図中の↑)，経時的に血中の抗HA抗体価をELISA法により測定した．抗体価は免疫前のサンプルよりも吸光度が0.1以上高い最大希釈倍率の逆数の対数をReciprocal $\log_2$ titerとして表した．文献9より作成．

C 針長800μmの皮膚内溶解型マイクロニードルパッチを成人健康男性の左上腕外側皮膚に1時間あるいは6時間貼付し，剥離後のマイクロニードルを実体顕微鏡により観察した．文献11より転載．

D 針長800μmの皮膚内溶解型マイクロニードルパッチを成人健康男性の左上腕外側皮膚に5秒間貼付し，剥離直後の貼付部位を共焦点レーザー生体顕微鏡により観察した．深さ1μm毎に撮影した画像を三次元構築することで，皮膚断面像を得た．文献11より転載．

とが示唆された．現在，皮膚内溶解型マイクロニードルを用いたインフルエンザ経皮ワクチンの臨床研究を実施しており，抗原装填マイクロニードルパッチがヒト皮膚に対して安全に適用でき，皮下注射とほぼ同等の抗体産生を誘導することを確認している．今後は，実用化に向けて，製剤製造法の樹立や品質試験法の確立など，製品化に焦点をおいた取り組みを推進していくことが重要であると考えている．

おわりに

皮膚に貼るだけで予防接種が可能なマイクロニードル製剤を用いた経皮ワクチンの研究開発に期待が寄せられており，筆者らは独自の皮膚内溶解型マイクロニードルを応用した経皮ワクチン製剤をすでに臨床研究へと展開し，ヒトにおける有用性を見出している．これらの研究成果は世界初のマイクロニードル型経皮ワクチンの実現に向けた大きな一歩であると考えられる．簡便性ならびに有効性に優れる経皮ワクチン製剤が実用化されれば，乳幼児へのワクチン接種の負担を大きく軽減できるのみならず，感染症地域への渡航者に対する事前予防ワクチンの施行，開発途上国へのワクチン普及に大きく貢献できるものと期待する．

参　考　文　献

1) Hirobe S, Okada N, Nakagawa S. Transcutaneous vaccines-current and emerging strategies. Expert Opin Drug Deliv 2013 ; 10 : 485-98.
2) Matsuo K, Hirobe S, Okada N, *et al.* Frontiers of transcutaneous vaccination systems : novel technologies and devices for vaccine delivery. Vaccine 2013 ; 31 : 2403-15.
3) Gerstel MS, Place VA. Drug delivery device. US Patent No. 3 1976 ; 964 : 482.
4) Kaushik S, Hord AH, Denson DD, *et al.* Lack of pain associated with microfabricated microneedles. Anesth Analg 2001 ; 92 : 502-4.
5) Van Damme P, Oosterhuis-Kafeja F, Van der Wielen M, *et al.* Safety and efficacy of a novel microneedle device for dose

sparing intradermal influenza vaccination in healthy adults. Vaccine 2009 ; 27 : 454-9.
6) Gill HS, Prausnitz MR. Coating formulations for microneedles. Pharm Res 2007 ; 24 : 1369-80.
7) Sullivan SP, Koutsonanos DG, Del Pilar Martin M, *et al.* Dissolving polymer microneedle patches for influenza vaccination. Nat Med 2010 ; 16 : 915-20.
8) Matsuo K, Yokota Y, Zhai Y, *et al.* A low-invasive and effective transcutaneous immunization system using a novel dissolving microneedle array for soluble and particulate antigens. J Control Release 2012 ; 161 : 10-7.
9) Matsuo K, Hirobe S, Yokota Y, *et al.* Transcutaneous immunization using a dissolving microneedle array protects against tetanus, diphtheria, malaria, and influenza. J Control Release 2012 ; 160 : 495-501.
10) Hiraishi Y, Nakagawa T, Quan YS, *et al.* Performance and characteristics evaluation of a sodium hyaluronate-based microneedle patch for a transcutaneous drug delivery system. Int J Pharm 2013 ; 441 : 570-9.
11) Hirobe S, Azukizawa H, Matsuo K, *et al.* Development and clinical study of a self-dissolving microneedle patch for transcutaneous immunization device. Pharm Res in press.

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A low-invasive and effective transcutaneous immunization system using a novel dissolving microneedle array for soluble and particulate antigens

Kazuhiko Matsuo [a], Yayoi Yokota [a], You Zhai [a], Ying-Shu Quan [b], Fumio Kamiyama [b], Yohei Mukai [a], Naoki Okada [a,*], Shinsaku Nakagawa [a,**]

[a] Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan
[b] CosMED Pharmaceuticals Co. Ltd, 448–5 Kajii-cho, Kamigyo-ku, Kyoto 602–0841, Japan

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ABSTRACT

Transcutaneous immunization (TCI) is a promising needle-free, easy-to-use, and low-invasive vaccination method. The hydrogel patch-based TCI system induced immune responses against soluble antigens (Ags) like toxoids, but could not induce immune responses against particulate Ags. Here, as an effective TCI system against every form of Ag, we developed a dissolving microneedle array of three lengths (200, 300, or 800 μm) made of hyaluronate as a novel TCI device. Unlike conventional microneedles, the microneedles of our dissolving microneedle arrays dissolved in the skin after insertion. Each dissolving microneedle array effectively delivered both soluble and particulate Ags under the stratum corneum. TCI using these dissolving microneedle arrays induced effective immune responses in rats regardless of the Ag form that were comparable to conventional vaccination using subcutaneous immunization. In addition, application of these dissolving microneedle arrays caused only slight skin irritation. These findings suggest that our TCI system can simply, safely, and effectively improve protective immune responses for every vaccine Ag.

1. Introduction

Vaccination is the most effective preventive measure for protecting against infection-related mortality. Most vaccines are administered by injection with syringes and needles. Conventional injectable vaccination, however, has several inherent problems; the requirement for medical personnel or techniques, needle-related disease or injuries, and storage or transport issues, such as maintaining a proper cold chain [1,2]. These factors prevent the widespread use of vaccines in developing countries and rapid mass vaccination on an emergency basis, such as during an emerging pandemic. The development of easy-to-use, needle-free, and low-invasive vaccination methods is an urgent task globally.

Transcutaneous immunization (TCI) offers an attractive vaccination method as it has the advantage of improved compliance over conventional injection systems [3,4]. Skin, the target site of TCI, has an advanced immune system with antigen-presenting cells (APCs) such as Langerhans cells (LCs) or dermal dendritic cells (dDCs) [5–8]. Direct antigen (Ag) delivery to these APCs could enhance immune responses. The upper layer of the skin, the stratum corneum, consists of corneocytes embedded in a highly organized crystalline lamellar structure of the intercellular lipid matrix, thereby creating a physical barrier to substance penetration [9,10].

Previously, to overcome this barrier, we developed a hydrogel patch and revealed that a TCI system using the hydrogel patch induces effective immune responses against tetanus and diphtheria toxoids [11–13]. The hydrogel patch, however, less effectively promotes particulate and insoluble Ag penetration through the stratum corneum, thus TCI using a hydrogel patch does not induce effective immune responses against such Ags. Most practical vaccine Ags are in a particulate state, such as a less virulent strain of bacteria. The development of a different TCI system that is effective against all Ag forms is needed.

A microneedle array contains many micrometer-sized needles that can create a transport pathway large enough for proteins and nanoparticles, but small enough to avoid pain [14]. In addition, microneedle arrays have the advantage that they penetrate the stratum corneum barrier to target immunocompetent cells in the skin, and the use of a disposable array is suitable for self-administration by the patient

Abbreviations: Ad, adenovirus vector; Ag, antigen; APC, antigen-presenting cell; DC, dendritic cell; dDC, dermal dendritic cell; ELISA, enzyme-linked immunosorbent assay; ELISPT, enzyme-linked immunospot; FITC, fluorescein isothiocyanate; HEK, human embryonic kidney; IDI, intradermal immunization; IFN, interferon; IL, interleukin; LC, Langerhans cell; OVA, ovalbumin; SCI, subcutaneous immunization; SP, silica particle; TCI, transcutaneous immunization; Th, helper T type; TS, tape-stripped.

* Correspondence to: N. Okada, Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan. Tel.: +81 6 6879 8176; fax: +81 6 6879 8176.
** Correspondence to: S. Nakagawa, Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179.
E-mail addresses: okada@phs.osaka-u.ac.jp (N. Okada), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

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[15]. This is a promising approach for a TCI system to deliver various types of antigens into the skin. Gerstel and Place first presented a microneedle system in a 1976 patent [16]. The first paper to demonstrate the use of microneedles for transdermal delivery was not published until 1998 [17], but since then microneedle arrays made from silicon, metal, stainless steel, or titanium have been reported. The clinical use of microneedle arrays has faced serious obstacles because needles on microneedle arrays can fracture and remain in the skin, which is a safety issue. In 2004, however, microneedle systems made with biocompatible or biodegradable polymers began to be developed [18]. These systems are superior to conventional microneedle systems with regard to safety, leading to their early clinical use. Moreover, there are now several microneedle designs, such as solid microneedles, hollow microneedles, and coated microneedles. In the present study, we developed a dissolving microneedle array as a novel TCI device. Our dissolving microneedle array is made from sodium hyaluronate, as the base material. As sodium hyaluronate is a component of skin tissue, it is safe for exogenous material insertion. The needles inserted into the skin eventually dissolve because of their ability to retain water. Because skin characteristics differ between animal species, we conducted our experiments in both mice and rats. We first evaluated our dissolving microneedle array as a TCI device, and investigated its ability to induce immune responses using model Ags, ovalbumin (OVA) as model soluble Ags, and adenovirus vector (Ad) as model particulate Ags.

2. Materials and methods

2.1. Animals and cell line

Female C57BL/6 mice (6 weeks old), female BALB/c mice (6 weeks old), female hairless rats (5 weeks old), and female Wistar ST rats (5 weeks old) were purchased from SLC Inc. (Hamamatsu, Japan). All animals were maintained in the experimental animal facility at the Osaka University and experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of Osaka University.

Human embryonic kidney (HEK) 293 cells, the helper cell line for adenoviral vector (Ad) propagation, were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

2.2. Vector

Replication-deficient Ad was based on the adenovirus serotype 5 backbone with deletions of the E1 and E3 regions. Ad-Luc, which expresses firefly luciferase under control of the cytomegalovirus promoter, was previously constructed using an improved *in vitro* ligation method [19]. Ad-Luc was propagated in HEK293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80 °C. Vector particle titers were evaluated spectrophotometrically using the method of Maizel et al. [20].

2.3. Hydrogel patch formulation

The hydrogel patch formulation was prepared as described previously [11–13]. The hydrogel patch formulation comprised cross-linked HiPAS™ acrylate medical adhesives (CosMED Pharmaceutical Co. Ltd., Kyoto, Japan): octyldodecyl lactate: glycerin: sodium hyaluronan = 100:45:30:0.2, as a weight ratio of composition.

2.4. Fabrication of dissolving microneedle arrays and vaccination procedure

The dissolving microneedle arrays containing Ags were fabricated by micromolding technologies with sodium hyaluronate as the base material. Briefly, sodium hyaluronate was dissolved in distilled water, and then Ags were added and uniformly mixed. The aqueous solution was cast into micromolds and dried in a desiccator at room temperature. The dissolving microneedle arrays were obtained by separating them from the molds. The microneedle arrays contained over 200 microneedles/cm^2 that were 200, 300, or 800 μm long, each with a distance of 600 μm between them. To form the microneedle transcutaneous patch system, arrays with an area of 0.8 cm^2 were fixed onto an adhesive film with a surface area of 2.3 cm^2. Henceforth, we describe our dissolving microneedle array as the MicroHyala (MH) with a needle length of 200 μm (MH200), 300 μm (MH300), and 800 μm (MH800).

The vaccination procedure is shown in Supplementary Fig. 1. Forty-eight hours before application of each MH, the back skin of animals except hairless rats was shaved using clippers, exposed by removing hair with depilatory cream, washed with water, and dried. All MHs were pressed on skin using a handheld applicator at 12.8 N/200 microneedles.

2.5. Dissolution kinetics analysis of microneedles on each MH

The MH200, MH300, and MH800 were applied to back skin of BALB/c mice or Wistar ST rat for 5, 15, 30, or 60 min (Supplementary Fig. 1). After removing the MH, the microneedles of each MH were photographed using stereoscopic microscopy to measure the length of the remaining.

2.6. In vivo skin irritation study

The MH200, MH300, and MH800 were applied to the back skin of Wistar ST rat for 30 min. To estimate skin irritation by application of each MH, the test sites were observed and scored for the signs of erythema or edema according to the Draize dermal scoring criteria [21] at 5 min, and 2, 6, 24, 48, or 72 h after removing each MH. Furthermore, to evaluate the degree of skin barrier dysfunction, the skin surface impedance between the MH application area and the non-application skin area was measured within 30 s using a Pocket Tester (CDM-03D; Custom Inc., Kanagawa, Japan) 5, 15, 30, 60, or 120 min after removing each MH.

2.7. Substance delivery into the skin by each MH

After the application of each MH containing fluorescein isothiocyanate (FITC)-labeled ovalbumin (FITC-OVA; 1 μg) or FITC-labeled amorphous silica particles (FITC-SP, particle size diameter: 300 μm, COREFRONT Co., Ltd., Tokyo, Japan; 1×10^9 particle) for the indicated duration, the skin was harvested, embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and frozen in liquid nitrogen. Frozen sections (10-μm thick) were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA), and then photographed using fluorescence microscopy (BZ-8000; Keyence Corporation, Osaka, Japan).

2.8. Vaccine protocol

To investigate the immune responses against (i) OVA (Sigma-Aldrich Inc., St. Louis, MO) as a model soluble Ag and (ii) Ad as a model particulate Ag, Wistar ST rats or hairless rats were vaccinated according to the following methods.

(i) OVA (1 μg)-containing MH200, MH300, and MH800 were prepared. These were applied to the back skin of C57BL/6 mice or Wistar ST rats for 6 h. As subcutaneous immunization (SCI) or intradermal immunization (IDI) groups, C57BL/6 mice or Wistar ST rats were subcutaneously or intradermally injected with OVA (1 μg). This procedure was repeated four times every 2 weeks.

(ii) The Ad (7.7×10^9 virus particle)-containing MH800 was prepared. This was applied to the back skin of hairless rats for 6 h. As control groups, hairless rats were subcutaneously injected with Ad (7.7×10^9 virus particle), or transcutaneously immunized with OVA (1 μg) using a hydrogel patch. This procedure was repeated three times every 2 weeks.

The MH was covered with wound management film (BIOCLUSIVE; Johnson & Johnson Medical, Ltd., Tokyo, Japan) to allow for better skin adherence.

2.9. Antibody titer measurement

Serum was collected from immunized mice or rats at the indicated time points, and the Ag-specific IgG titer was determined by enzyme-linked immunosorbent assay (ELISA) following the previously described protocols [12]. End-point titers of the Ag-specific antibody were expressed as the reciprocal $\log_2$ of the last dilution that had 0.1 absorbance units after subtracting the background.

2.10. ELISPOT assay

C57BL/6 mice were transcutaneously, subcutaneously, or intradermally vaccinated with 100 μg OVA three times at 2-week intervals. Two weeks after the final vaccination, single-cell suspensions of lymph node cells or splenocytes were isolated from the mice and seeded onto 96-well plates at 4.0×10^5 cells/well. After 24-h stimulation with 1 mg/ml OVA, the frequency of interferon (IFN)-γ and interleukin (IL)-4-secreting cells specific for OVA was evaluated using a mouse IFN-γ or IL-4 ELISPOT kit (Mabtech AB, Nacka Strand, Sweden) according to the manufacturer's protocol.

2.11. Inhibitory experiment for Ad infection

Two weeks after the final vaccination, hairless rats vaccinated three times with Ad were intravenously injected with Ad-Luc at vector particle titers of 10^9. Two days later, the liver was removed from these hairless rats, weighed, and homogenized in phosphate-buffered saline containing 10 μg/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride. Luciferase activity in the homogenates was determined using a luciferase assay system (Promega, Madison, WI).

3. Results

3.1. Fabrication and characteristics of each MH as a TCI device

We successfully fabricated three types of dissolving microneedle arrays made of sodium hyaluronate and in various forms and lengths: MH200, MH300, and MH800 (Fig. 1A). We examined the microneedles dissolution process at different times after skin insertion. For the MH200 and MH300 applied on both mice and rats, the microneedle tips dissolved within 5 min and were fully dissolved at 1 h (Fig. 1B,C). For the MH800, the microneedles were reduced in length by 50% within 5 min, and had completely dissolved by 1 h. These data showed microneedles surely dissolved in skin of animals with different thick stratum corneum. Thus, our TCI system using the MH should be applied for at least 1 h.

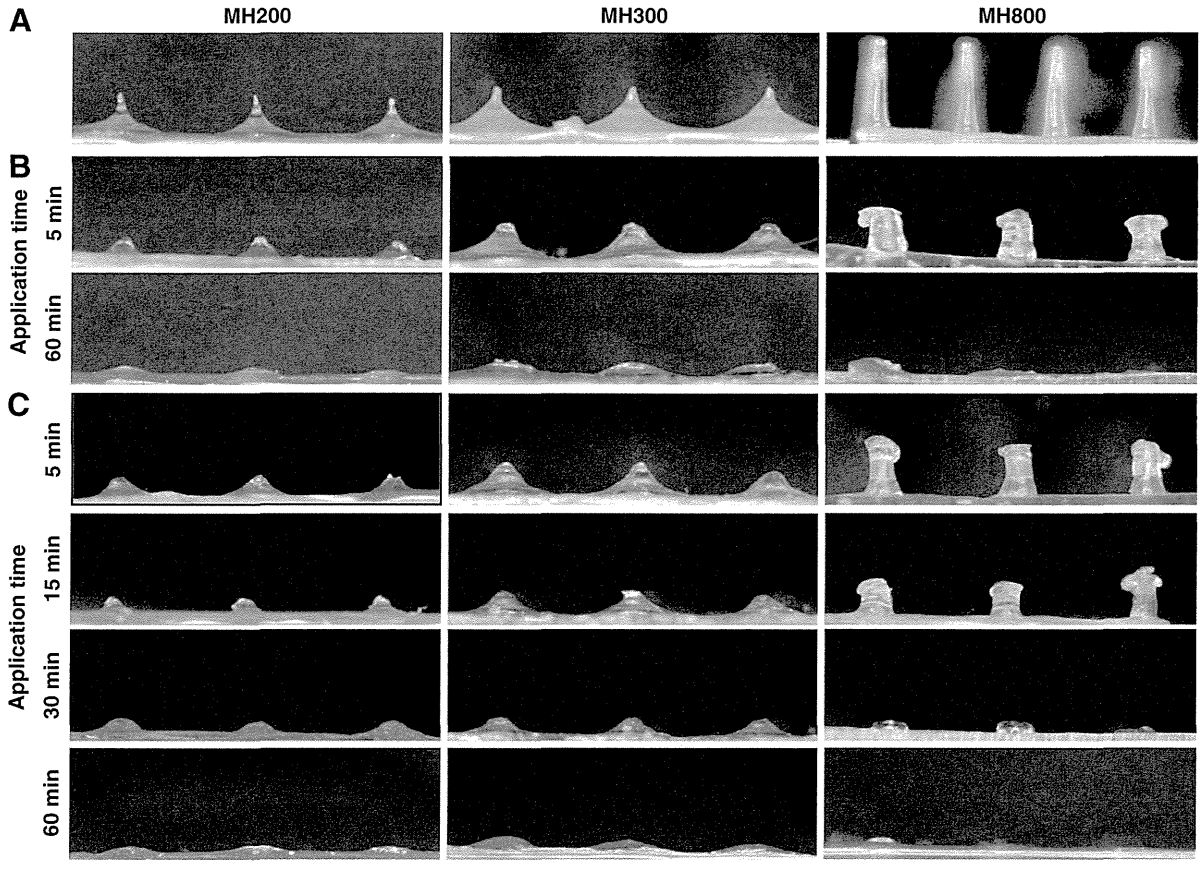

Fig. 1. Micrograph of dissolving microneedle arrays and dissolution kinetics of microneedles of each MH after skin insertion. (A) Bright-field micrograph of microneedles on MH200, MH300, and MH800. (B, C) The MH200, MH300, or MH800 was applied on the back skin of BALB/c mice (B) or Wistar ST rats (C) for 5, 15, 30, or 60 min. After removal of the MH, the microneedles remaining on each MH were photographed under a stereoscopic microscope.

3.2. Skin irritation caused by the application of each MH

We evaluated erythema and edema at the application site using the Draize scoring system after 30-min application of each MH (Fig. 2A). The skin of all rats showed no edema or erythema before application (data not shown). After 30 min application of each MH, edema was not observed in any rats. The MH800 induced slight or moderate erythema that gradually disappeared within a few days (Fig. 2B). The MH200 and MH300 induced slight erythema that disappeared within a few hours. In addition, we measured the skin impedance to evaluate the skin barrier dysfunction caused by each MH (Fig. 2C). Application of each MH decreased skin impedance immediately after removal: MH800 (20%) > MH200 (60%) > MH300 (70%), but these values recovered within 2 h. On the other hand, on tape-stripped skin in which the stratum corneum was physically removed, the relative skin electric impedance was decreased to 20% and did not recover within 2 h. This result suggests that the holes caused by insertion of each MH closed up quickly. Thus, application of each MH caused only temporary skin irritation, indicating that our dissolving microneedle arrays are low-invasive TCI devices.

3.3. Localization of antigens delivered by each MH

We evaluated whether the MH could deliver not only soluble Ags but also particulate Ags into the skin using FITC-OVA (soluble substance) and FITC-SP (particulate substance). For mice, MH200 delivered FITC-OVA (green spot) or FITC-SP (green spot) into the epidermis and upper layer of the dermis, whereas MH300 and MH800 delivered them into mainly the dermis (Fig. 3A). On the other hand, for rats, MH200 and MH300 delivered FITC-OVA or FITC-SP into the epidermis and upper dermis layer, whereas MH800 delivered them from the epidermis to the lower dermis layer in the skin (Fig. 3B). These results indicated that the MH delivered various Ags into the skin regardless of the Ag form. In addition, the MH size can be used to control the Ag delivery site.

3.4. Characterization of immune responses induced by TCI using the Ag-encapsulating MHs

We investigated whether our TCI system using each MH induced Ag-specific IgG production against OVA as a model soluble Ag. TCI using each MH increased anti-OVA IgG titer in the sera of both C57BL/6 mice and Wistar ST rats (Fig. 4A,B). The effect tends to be equal or superior to that of SCI or IDI group. Additionally, in comparison of antibody production between TCI using MH200, MH300, and MH800, for C57BL/6 mice, anti-OVA IgG titer induced by TCI using MH300 or MH800 was higher than that of MH200 (Fig. 4a). On the other hand, Wistar ST rats vaccinated once with MH800 showed higher OVA-specific IgG production than that in the TCI

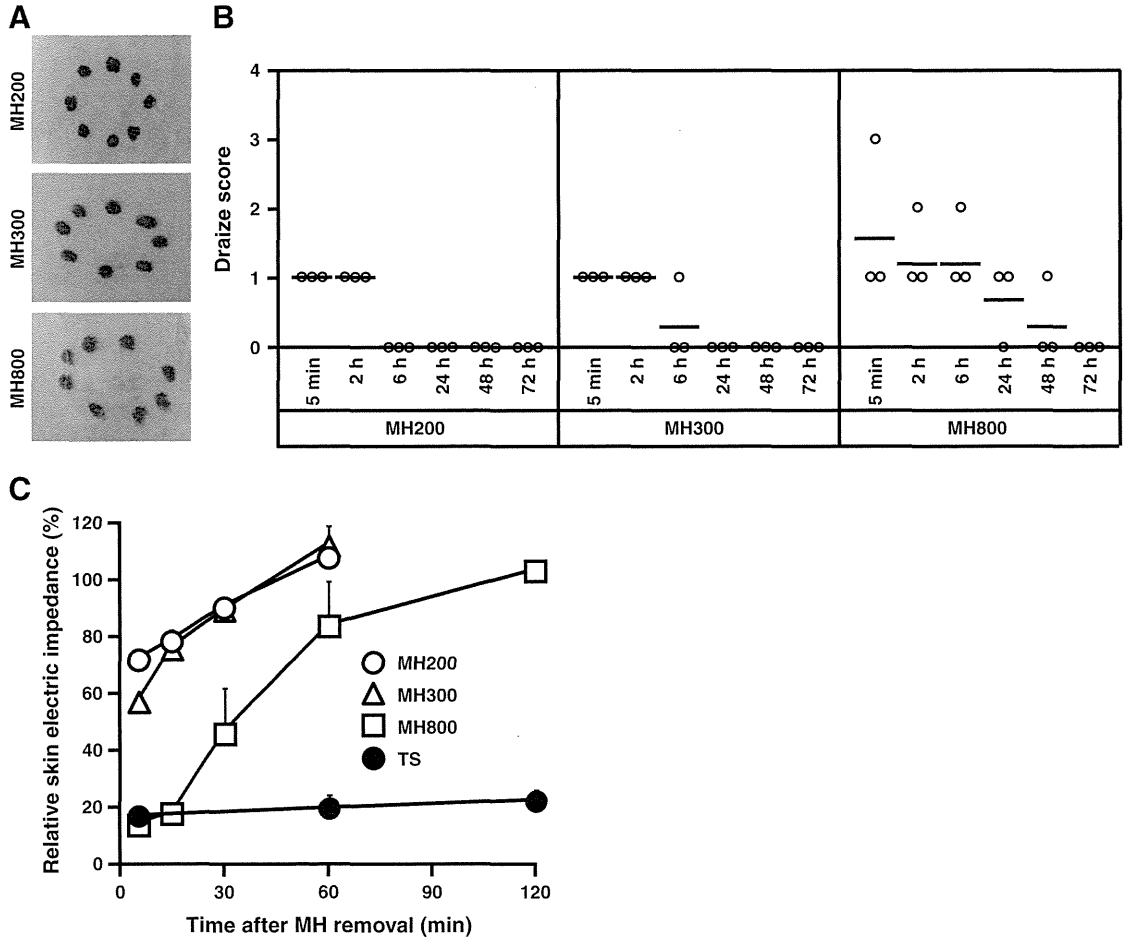

Fig. 2. Assessment of skin irritation caused by application of each MH. The MH200, MH300, or MH800 was applied to the back skin of Wistar ST rats for 30 min. (A) After MH removal, the application site was observed. (B) The degree of erythema on the skin of Wistar ST rats was scored using the Draize scoring system: 0, no erythema or edema; 1, very slight erythema and/or barely perceptible edema; 2, well-defined erythema and/or slight edema; 3, moderate to severe erythema or moderate edema, and 4, severe erythema and/or edema. Mean value is shown as a bar. (C) Skin impedance between the MH application area and non-application area was measured 5, 15, 30, 60, and 120 min after the removal of each MH. As the control group, the back skin of Wistar ST rats was tape-stripped. Data are expressed as mean ± SE of results from three rats. TS, tape-stripped, TCI; transcutaneous immunization. In each section, the stratum corneum lies between the top line and the middle line, the living epidermis lies between the middle line and bottom line, and the dermis lies under the bottom line.

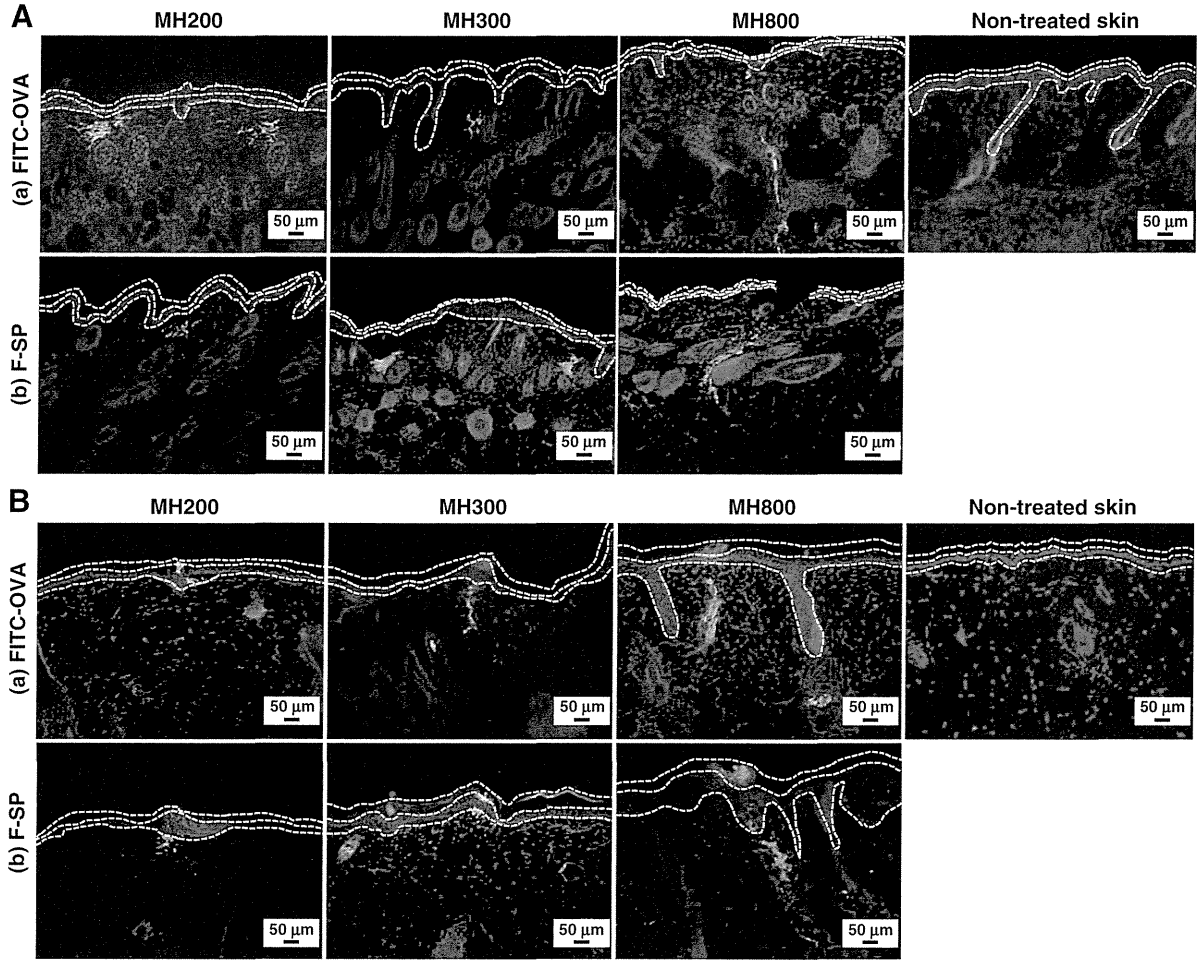

Fig. 3. Skin sections from animals vaccinated with each MH encapsulating FITC-OVA or FITC-SP. FITC-OVA (green) or FITC-SP (green)-containing MH200, MH300, and MH800 were applied on the back skin of C57BL/6 mice (A) or Wistar ST rats (B) for 6 h. The skin was harvested and frozen. Frozen sections (6-μm thick) were photographed under a fluorescence microscope. The nucleus was counterstained using DAPI (blue).

groups using MH200 and MH300, but the anti-OVA IgG titer reached maximum levels by the fourth vaccination in all TCI groups (Fig. 4B).

To evaluate the Th1/Th2 balance in the immune responses induced by our TCI, we analyzed the OVA-specific IgG subclass. On the analysis of IgG subclass in C57BL/6 mice, OVA injection induced mainly anti-

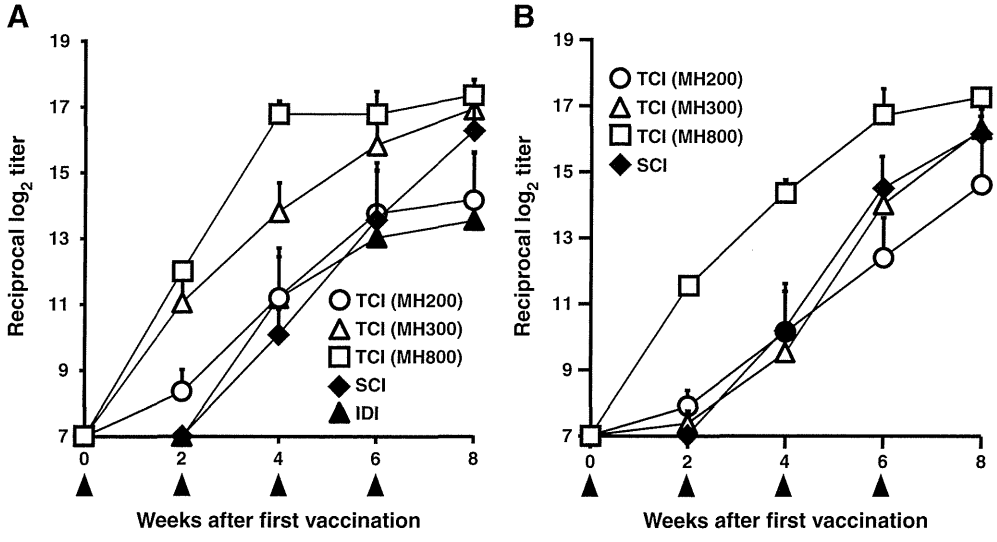

Fig. 4. OVA-specific antibody responses after transcutaneous vaccination. C57BL/6 mice (A) or Wistar ST rats (B) were transcutaneously vaccinated with 1 μg OVA using MH200, MH300, or MH800 for 6 h four times at 2-week intervals. A control group was subcutaneously or intradermally immunized with 1 μg OVA. At the indicated points, sera collected from these mice or rats were assayed for the OVA-specific IgG titer by ELISA. TCI; transcutaneous immunization, SCI; subcutaneous immunization, IDI; intradermal immunization. Arrowhead indicates vaccination point.

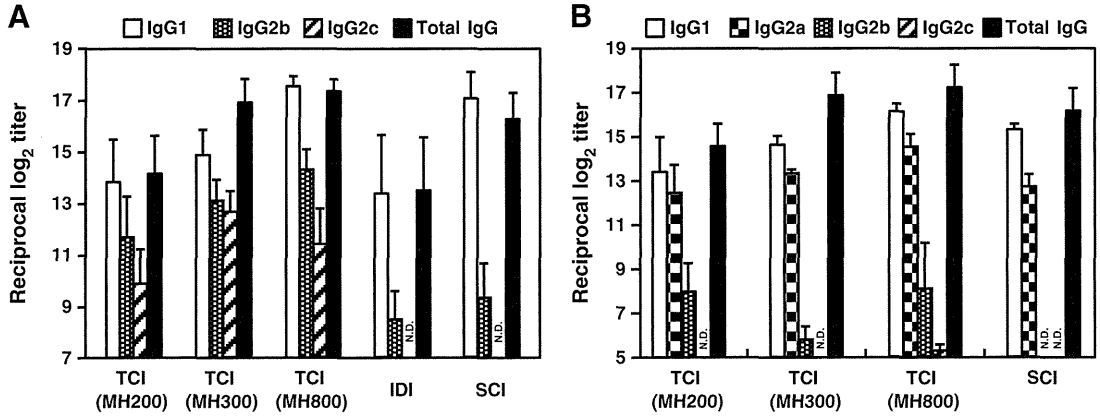

Fig. 5. Analysis of OVA-specific IgG subclass. An OVA (1 μg)-encapsulated MH200, MH300, or MH800 were applied to the back skin of C57BL/6 mice (A) or Wistar ST rats (B) for 6 h four times at 2-week intervals. A control group was subcutaneously or intradermally immunized with 1 μg OVA. Two weeks after the final vaccination, serum collected from these animals was assayed for OVA-specific IgG subclass (IgG1, IgG2a, IgG2b, IgG2c) titer by ELISA. Data are expressed as mean ± SE of results from 5 animals. ND; not detectable, TCI; transcutaneous immunization, SCI; subcutaneous immunization, IDI; intradermal immunization.

OVA IgG subclass IgG1 (Th2-type IgG subclass) not IgG2c (Th1-type IgG subclass), whereas TCI using MH200, MH300, and MH800 induced both IgG1 and IgG2c specific for OVA (Fig. 5A). On the other hand, in Wistar ST rats vaccinated by TCI using each MH, mainly IgG1 and IgG2a (Th2-type subclass) not IgG2c (Th1-type subclass) were increased, as well as in SCI group (Fig. 5B). TCI using MHs induced distinct immune responses between C57BL/6 mice and Wistar ST rats. The cause of these results might require further investigation.

Since, in C57BL/6 mice, interferon (IFN)-γ (Th1-type cytokine) and interleukin (IL)-4 (Th2-type cytokine) induce a class switch to IgG1 and IgG2c, we analyzed IFN-γ and IL4-secreting lymphocyte cells upon stimulation of OVA. In ELISPOT assay, the frequency of IFN-γ-secreting cells and IL-4-secreting cells in both lymph node cells and splenocytes of C57BL/6 mice vaccinated with OVA by TCI using MH200, MH300, or MH800 were detected fewer than that of SCI group (Fig. 6). This result contradicted above data of IgG subclass analysis. Since various immune events might contribute Th1/Th2 immune balance, to understand the characteristics of immune responses induced by TCI using each MH in detail, we need to investigate Ag-capturing APC subsets and the Ag-presenting function of the APC subsets.

3.5. Protective efficacy against particulate antigen by TCI using Ag-encapsulating MHs

Furthermore, to examine whether our TCI system induced immune responses against particulate Ags, we evaluated the vaccine efficacy of the TCI using the MH800 against Ad as a particulate model Ag (Fig. 7A). TCI using MH800 increased the Ad-specific IgG

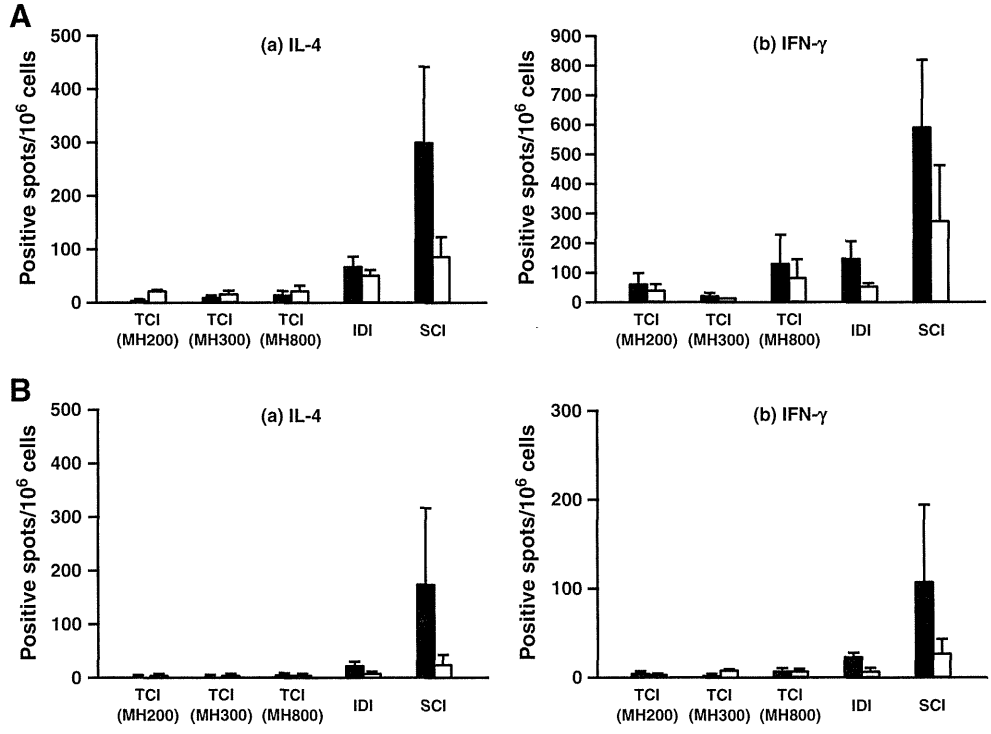

Fig. 6. Cytokine production in lymphoid tissue of mice vaccinated transcutaneously with OVA. C57BL/6 mice were transcutaneously vaccinated with 1 μg OVA using MH200, MH300, or MH800 four times at 2-week intervals. As control groups, C57BL/6 mice were intradermally or subcutaneously immunized with 1 μg OVA. Two weeks after the final vaccination, single-cell suspensions of draining lymph node cells (A) or splenocytes (B) were prepared and an ELISPOT assay was performed after stimulating the cells with (■) or without (□) 1 mg/ml OVA for 24 h. Data are expressed as mean ± SE of results from five mice. TCI; transcutaneous immunization, SCI; subcutaneous immunization, IDI; intradermal immunization.

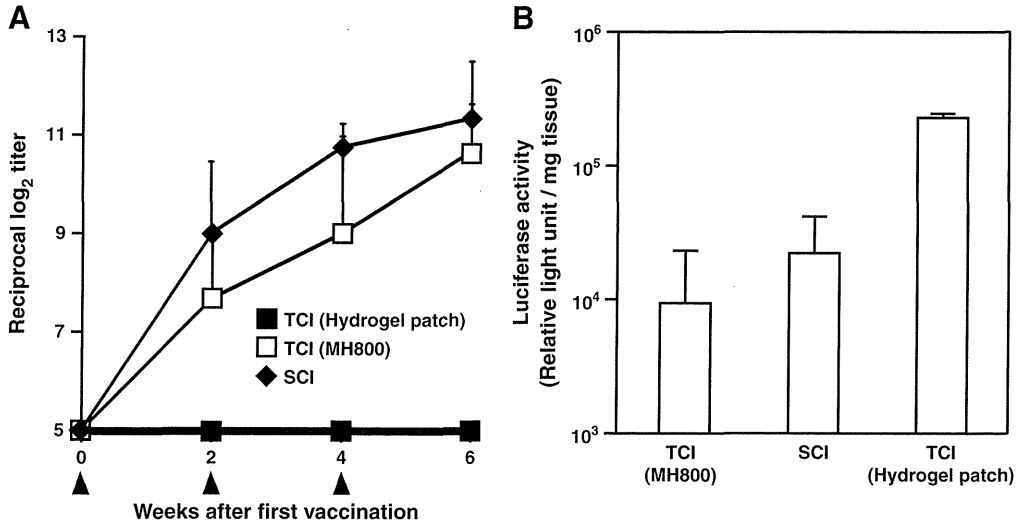

Fig. 7. Inhibitory effects for Ad infection in hairless rats vaccinated transcutaneously with Ad. Ad (7.7×10^9 VP) was applied on the back skin of hairless rats using an MH800 for 6 h, or using a hydrogel patch for 24 h at 2-week intervals. A control group was subcutaneously immunized with Ad (7.7×10^9 VP). (A) At the indicated points, sera collected from these rats were assayed for the Ad-specific IgG titer by ELISA. (B) Two weeks after the final vaccination, hairless rats vaccinated with Ad were intravenously injected with Ad-Luc at vector particle titers of 10^9. Two days later, luciferase activity in liver homogenates was determined using a luciferase assay system. Data are expressed as mean ± SE of 3 to 5 rats. Arrowheads indicate the vaccination point. TCI; transcutaneous immunization, SCI; subcutaneous immunization.

titer after the first vaccination. This effect was equal to that of the SCI group. On the other hand, hairless rats vaccinated with TCI using a hydrogel patch showed no Ad-specific IgG antibody production, because the hydrogel patch could not promote particulate Ag penetration through the stratum corneum. Next, to evaluate the neutralization activity of the anti-Ad IgG antibody, we intravenously injected into hairless rats 2 weeks after the final vaccination. Two days later, luciferase activity in the liver of hairless rats vaccinated transcutaneously using the MH800 was repressed compared with that of TCI using a hydrogel patch, comparable to that of the SCI group (Fig. 7B). Thus, TCI using the MH induced neutralizing antibodies against particulate antigens.

Based on these results, our TCI system using the MH induced immune responses against particulate Ags as well as soluble Ags, suggesting that our original device would be applicable to every type of vaccine Ag.

4. Discussion

In the present study, we developed our original TCI system using dissolving microneedle arrays; the MH made of sodium hyaluronate, which was fabricated in various forms and lengths; MH200, MH300, and MH800. The microneedles on the MH were dissolved by water in the skin and thus had no danger of remaining in the skin, making our MH safer than traditional microneedle arrays made of metal or stainless steel. In fact, the condition of the skin on which each MH was applied recovered immediately after MH removal.

In both animal species, mice and rats, each MH showed similar insertion characteristics and induction of immune response, suggesting that our MHs dissolved in the skin and delivered Ags into skin with various characteristics. Our results suggested that the MH can be developed for clinical use, because it will be suitable for insertion into people of different races with different skin characteristics all over the world. It is reported that the force required for the insertion of microneedles into skin is over 0.058 N/needle [18]. As the microneedles on each MH had enough force for insertion (data not shown), application to human skin is promising.

In addition, TCI using each MH induced Ag-specific immune responses. This result is due to the ability of Ag to be delivered by each MH into a living epidermis and dermis in which APCs are present. This is important information to advance the development of each MH. Human skin characteristics vary (skin thickness, skin turgor, and so on) with those of rodent models, thus we are now investigating the characteristics of Ag delivery and microneedle insertion in a human skin model.

Furthermore, TCI using the each type of MH induced immune responses against both soluble and particulate Ags, suggesting that this system is applicable to not only soluble Ags like toxoids, but also to inactivated whole-organism vaccines and live vaccines. In this respect, further studies of the activity retention of Ags contained in the MH are needed.

In addition, there were difference in strength of Ag-specific antibody production induced by TCI using MH200, MH300, and MH800 in both mice and rats. This distinct immune response seemed to be due to differences in the APC subset involved in Ag capture. The APC subset differs in each layer of the skin, such as LCs in living epidermis [22] or dDC in dermis [23]. If Ags were delivered to different skin layers, then distinct immune responses would be induced. In mice, Ags administrated into living epidermis and part of dermis by TCI using MH200 are captured mainly by LCs, whereas LCs as well as dDCs captured Ags delivered into both living epidermis and dermis by TCI using MH300 and MH800. On the other hand, in rats, Ags might be delivered into living epidermis and part of dermis by TCI using MH200 and MH300, and are thus captured mainly by LCs. Also, in rats vaccinated by TCI using MH800, LCs and dDCs capture Ags delivered into both living epidermis and dermis. Though the distinct immune function mechanism between LCs and dDCs is not completely clear, dDCs might migrate into regional lymph nodes more quickly than LCs in Ag administration [24]. We are currently working to identify the Ag-capturing APC subsets in TCI using each MH and to analyze the *in vivo* kinetics of these cells.

In addition, dDC were recently classified into two subsets; langerin (C-type lectin receptor; CD207)-positive dDC, or langerin-negative dDC [25–27]. The immune responses induced by these APC subsets in the skin, such as LC, langerin-positive dDC, or langerin-negative dDC, have not been investigated *in vivo*. Because the MH might deliver the Ags into particular skin layers, such as living epidermis and upper or lower layers of the dermis, the Ag can be selectively delivered to specific APC subsets, suggesting that it is possible to analyze the *in vivo* mechanism of immune function induced by these APC subsets. On the basis of these analyses, we will clarify the skin immune system and also discuss the detail immune characteristics of our TCI system. We are currently analyzing the association between Ag delivery site,

Ag-capturing cell groups, and immune responses by TCI using the MH200, MH300, and MH800.

5. Conclusion

These data indicated that our MH, as a novel TCI device, delivered Ags into the skin regardless of the Ag form. Therefore, TCI using the MH safely and effectively induced immune responses against soluble and particulate Ags. The application of our TCI system against practical infectious disease, such as influenza, is now being evaluated. In addition, we are currently working to analyze the mechanism of immune responses induced by our TCI.

Supplementary materials related to this article can be found online at doi:10.1016/j.jconrel.2012.01.033.

Acknowledgments

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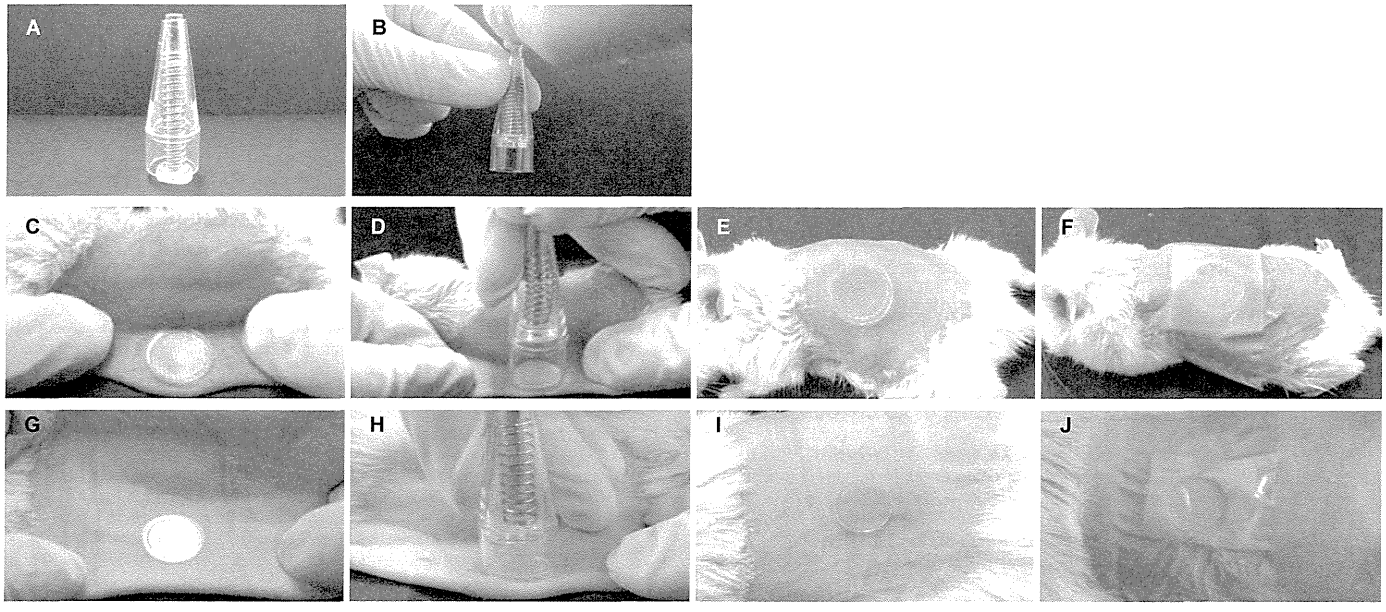
References

[1] G. Kersten, H. Hirschberg, Needle-free vaccine delivery, Expert Opin. Drug Deliv. 4 (5) (2007) 459–474.
[2] N. Azad, Y. Rojanasakul, Vaccine delivery-current trends and future, Curr. Drug Deliv. 3 (2) (2006) 137–146.
[3] G.M. Glenn, D.C. Flyer, L.R. Ellingsworth, S.A. Frech, D.M. Frerichs, R.C. Seid, J. Yu, Transcutaneous immunization with heat-labile enterotoxin: development of a needle-free vaccine patch, Expert Rev. Vaccines 6 (5) (2007) 809–819.
[4] T. Warger, H. Schild, G. Rechtsteiner, Initiation of adaptive immune responses by transcutaneous immunization, Immunol. Lett. 109 (1) (2007) 13–20.
[5] A.R. Mathers, A.T. Larregina, Professional antigen-presenting cells of the skin, Immunol. Res. 36 (1–3) (2006) 127–136.
[6] K. Sugita, K. Kabashima, K. Atarashi, T. Shimauchi, M. Kobayashi, Y. Tokura, Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T cells in the skin, Clin. Exp. Immunol. 147 (1) (2007) 176–183.
[7] C.L. Berger, J.G. Vasquez, J. Shofner, K. Mariwalla, R.L. Edelson, Langerhans cells: mediators of immunity and tolerance, Int. J. Biochem. Cell Biol. 38 (10) (2006) 1632–1636.
[8] N. Romani, B.E. Clausen, P. Stoitzner, Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin, Immunol. Rev. 234 (1) (2010) 120–141.
[9] B.W. Barry, Breaching the skin's barrier to drugs, Nat. Biotechnol. 22 (2) (2004) 165–167.
[10] J.M. Jensen, E. Proksch, The skin's barrier, G. Ital. Dermatol. Venereol. 144 (6) (2009) 689–700.
[11] Y. Ishii, T. Nakae, F. Sakamoto, K. Matsuo, K. Matsuo, Y.S. Quen, F. Kamiyama, T. Fujita, A. Yamamoto, S. Nakagawa, N. Okada, A transcutaneous vaccination system using a hydrogel patch for viral and bacterial infection, J. Control. Release 131 (2) (2008) 113–120.
[12] K. Matsuo, Y. Ishii, Y.S. Quen, F. Kamiyama, Y. Mukai, Y. Yoshioka, N. Okada, S. Nakagawa, Transcutaneous vaccination using a hydrogel patch induces effective immune responses to tetanus and diphtheria toxoid in hairless rat, J. Control. Release 149 (1) (2011) 15–20.
[13] K. Matsuo, Y. Ishii, Y.S. Quen, F. Kamiyama, Y. Mukai, Y. Yoshioka, N. Okada, S. Nakagawa, Characterization of transdermal delivery by s hydrogel patch in animals, human, and tissue-engineered skin model, Biol. Pharm. Bull. 34 (4) (2011) 586–589.
[14] S. Henry, D.V. McAllister, M.G. Allen, M.R. Prausnitz, Microfabricated microneedles: a novel approach to transdermal drug delivery, J. Pharm. Sci. 88 (9) (1999) 948.
[15] M.R. Prausnitz, Microneedles for transdermal drug delivery, Adv. Drug Deliv. Rev. 56 (5) (2004) 581–587.
[16] M.S. Gerstel, V.A. Place, Drug Delivery Device, US Patent No. 3, 964 (1976) 482.
[17] S. Henry, D.V. McAllister, M.G. Allen, M.R. Prausnitz, Microfabricated microneedles: a novel approach to transdermal drug delivery, J. Pharm. Sci. 87 (8) (1988) 922–925.
[18] J.H. Park, M.G. Allen, M.R. Prausnitz, Biodegradable polymer microneedles: fabrication, mechanics and transdermal, drug delivery, 104 (1) (2005) 51–66.
[19] H. Mizuguchi, M.A. Kay, Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method, Hum. Gene Ther. 9 (17) (1998) 2577–2583.
[20] J.V. Maizel Jr., D.O. White, M.D. Scharff, The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12, Virology 36 (1) (1968) 115–125.
[21] J.H. Draize, G. Woodard, H.O. Calvery, Methods for the study of irritaion and toxicity of substances applied topically to the skin and mucous membranes, J. Pharmacol. Exp. Ther. 82 (1944) 377–390.
[22] N.C. Smith, L. Favila-Castillo, A. Monroy-Ostria, C. Hirunpetcharat, M.F. Good, The spleen, IgG antibody subsets and immunity to Plasmodium berghei in rats, Immunol. Cell Biol. 75 (3) (1997) 318–323.
[23] D. Sen, L. Forrest, I. Parker, M.D. Cahalan, Selective and site-specific mobilization of dermal dendritic cells and Langerhans cells by Th1- and Th2-polarizing adjuvants, Proc. Natl. Acad. Sci. 107 (18) (2010) 8334–8339.
[24] L.S. Bursch, L. Wang, B. Igyarto, A. Kissenpfennig, B. Malissen, D.H. Kaplan, K.A. Hogquist, Identification of a novel population of Langerin+ dendritic cells, J. Exp. Med. 204 (13) (2007) 3147–3156.
[25] F. Ginhoux, M.P. Collin, M. Bogunovic, M. Abel, M. Leboeuf, J. Helft, J. Ochando, A. Kissenpfennig, B. Malissen, M. Grisotto, H. Snoeck, G. Randolph, M. Merad, Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state, J. Exp. Med. 204 (13) (2007) 3133–3146.
[26] L.F. Poulin, S. Henri, B. de Bovis, E. Devilard, A. Kissenpfennig, B. Malissen, The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells, J. Exp. Med. 204 (13) (2007) 3119–3131.
[27] M. Merad, F. Ginhoux, M. Collin, Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells, Nat. Rev. Immunol. 8 (12) (2008) 935–947.



Supplementary Fig. 1. Schematic drawing of transcutaneous vaccination procedure. A and B; applicator for MH application, C, D, E, G, H, and I; application of MH to back skin of mice (C,D,E) or rats (G,H,I), F and J; covering with a wound management film over MH.

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Corrigendum

Corrigendum to "A low-invasive and effective transcutaneous immunization system using a novel dissolving microneedle array for soluble and particulate antigens" [J. Control. Release 161 (2012) 10–17]

Kazuhiko Matsuo [a], Yayoi Yokota [a], You Zhai [a], Ying-Shu Quan [b], Fumio Kamiyama [b], Yohei Mukai [a], Naoki Okada [a,*], Shinsaku Nakagawa [a,**]

[a] Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] CosMED Pharmaceuticals Co. Ltd, 32 Higashikujokawanishi-cho, Minami-ku, Kyoto 601-8014, Japan

The authors regret that several errors appeared in the figure legends and acknowledgment of the original publication, therefore the correct related texts appear below.

Materials and methods

2.1. Animals and cell line (page 11)

First sentence of this section should be changed to "Female ICR mice (6 weeks old), female C57BL/6 mice (6 weeks old), female BALB/c mice (6 weeks old), female hairless rats (5 weeks old), and female Wistar ST rats (5 weeks old) were purchased from SLC Inc. (Hamamatsu, Japan)."

2.7. Substance delivery into the skin by each MH (page 11)

Revised full sentence of this section is as follows:

After the application of each MH containing fluorescein-conjugated ovalbumin (F-OVA; 1 μg) or fluorescein isothiocyanate (FITC)-labeled amorphous silica particles (FITC-SP, particle size diameter: 300 μm, COREFRONT Co., Ltd., Tokyo, Japan; 1×10^9 particle) for 1 h to the back skin of ICR mice or Wistar ST rats, the skin was harvested, embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and frozen in liquid nitrogen. Frozen sections (8-μm thick) were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA), and then photographed using fluorescence microscopy (BZ-8000; Keyence Corporation, Osaka, Japan)

Figures

Fig. 2 (page 13)

Last sentence of Fig. 2 legend "In each section, the stratum corneum lies between the top line and the middle line, the living epidermis lies between the middle line and bottom line, and the dermis lies under the bottom line" should be deleted.

Fig. 3 (page 14)

Fig. 3 legend should read as "Skin sections from animals vaccinated with each MH encapsulating F-OVA or FITC-SP. F-OVA (green) or FITC-SP (green)-containing MH200, MH300, and MH800 were applied on the back skin of ICR mice (A) or Wistar ST rats (B) for 1 h. The skin was harvested and frozen. Frozen sections (8-μm thick) were photographed under a fluorescence microscope. The nucleus was counterstained using DAPI (blue). In each section, the stratum corneum lies between the top line and the middle line, the living epidermis lies between the middle line and bottom line, and the dermis lies under the bottom line."

Acknowledgments (page 17)

Acknowledgements section should read as "This investigation was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO); Health Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare; a Grant-in-Aid for challenging Exploratory Research (No. 22659033) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a Grant-in-Aid from the Uehara Memorial Foundation; a Grant-in-Aid from The Mochida Memorial Foundation for Medical and Pharmaceutical Research; and a Grant-in-Aid from the Tokyo Biochemical Research Foundation."

Note

The corrections made in this corrigendum do not affect the original conclusion.

The authors would like to apologize for any inconvenience caused.

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* Correspondence to: N. Okada, Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel./fax: +81 6 6879 8176.

** Correspondence to: S. Nakagawa, Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179.

E-mail addresses: okada@phs.osaka-u.ac.jp (N. Okada), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

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