表1　歯科金属アレルギーにより惹起された皮膚粘膜疾患

舌炎、口内炎、口唇炎、歯肉口唇炎、肉芽腫口唇炎、蕁麻疹、クインケ浮腫、口腔内扁平苔癬、皮膚扁平苔癬、皮膚そう痒症、浮腫性紅斑、掌蹠膿疱症、汗疱状皮膚炎、好酸球性膿疱性毛包炎、貨幣状湿疹、全身の皮膚炎（pseudo-atopic dermatitis）

中山秀夫他：歯界展望、1974より引用

（カルバニズム）が生じ，電気化学的に金属の溶出が起こると考えられる[3]。

2. 臨床型

金属の接触アレルギーは，局所型と全身型の二つのタイプが存在する。ネックレス，ピアス，イヤリング，指輪などの装飾品，時計，皮革製品，コイン，セメントや消毒薬などに含まれている金属が直接接触し皮膚に炎症を起こすタイプを局所型の金属接触アレルギーといい，食品，歯科金属や骨接合用金属に含まれている微量金属が，経口摂取や経気道吸収などの経皮吸収以外の経路により体内に吸収された金属によって惹起されるタイプを，全身型の金属接触アレルギーという。

Fisherは，接触感作が成立した固体に，原因アレルゲンが経皮的以外の経路で吸収されて発症するものをsystemic contact-type dermatitisと定義している[4]。全身型接触アレルギーの発症様式がこれにあてはまる。中山らが提唱する歯科金属疹[5]も全身型接触アレルギーである。

歯科金属疹は，皮疹型から①汗疱型，②パッチテスト部位の再燃現象，③汎発性紅斑丘疹小水疱，④多形紅斑および血管炎，⑤蕁麻疹の5型に分類されている[4,6]。

この発疹型の中で最も頻度が高いのが汗疱型であり，汗疱状湿疹，異汗性湿疹と診断される。

3. 診断

1）問診

金属による局所型の接触アレルギーは，患者が自覚している場合が多いが，長期間口腔内に存在している歯科金属に関しては，口粘膜に所見や自覚症状の存在することは稀である。また一般的に，長期間口腔内に存在している歯科金属はアレルゲンになりにくいと思われている。そこでその診断には，異味症，口腔内異和感，歯科治療歴や口臭の有無など詳細な問診をとることが必要である。当科では，口腔内のどの位置に何色の歯科金属が装着されているかをスケッチしている。黄金色をしていなくても合金として金が含有されている場合がある。また，慢性，難治性の経過をたどる扁平苔癬や貨幣状湿疹の中には，金属アレルギーが原因である場合もある。このように歯科金属アレルギーにより惹起される皮膚粘膜疾患には，扁平苔癬や貨幣状湿疹以外に表1に示すものなどがある。

2）パッチテスト

金属アレルギーの検出法として，パッチテストは侵襲が少なく簡便で有用な方法であ

表2　Japanese standard allergens

1	Cobalt chloride	1.0%pet.	15	Lanolin alcohol	30.0%pet.
2	Nickel sulfate	2.5%pet.	16	PTBP-FR	1.0%pet.
3	Potassium dichromate	0.5%pet.	17	Ethylenediamin 2HCl	1.0%pet.
4	Thiuram mix	1.25%pet.	18	Primin	0.01%pet.
5	PPD black rubber mix	0.6%pet.	19	Urushiol	0.02%pet.
6	Mercapto mix	2.0%pet.	20	Thimerosal	0.1%pet.
7	Caine mix	7.0%pet.	21	Ammoniated mercury	1.0%pet.
8	Fradiomycin sulfate	20.0%pet.	22	Petrolatum	as is
9	Balsam of Peru	25.0%pet.	23	Formaldehyde	1.0%aq.
10	Rosin	20.0%pet.	24	Kathon CG	0.01%aq.
11	Fragrance mix	8.0%pet.	25	Gold sodium thiosulfate	0.5%pet.
12	Dithiocarbamate mix	2.0%pet.	26	Thimerosal	0.05%pet.
13	Paraben mix	15.0%pet.	27	Mercuric chloride	0.05%aq.
14	Paraphenylenediamine	1.0%pet.	28	Distilled water	as is

pet.:petrolatum　　PTBP-FR: p-tert-butylphenol formaldehyde resin
aq.:aqueous solution

表3　金属シリーズ

1	塩化第二鉄	2.0%aq..	11	塩化マンガン	0.5%pet.
2	塩化第二スズ	1.0%aq.	12	金チオ硫酸ナトリウム	0.5%pet.
3	四塩化イリジウム	1.0%aq.	13	重クロム酸カリウム	0.5%pet.
4	三塩化インシジウム	1.0%aq.	14	塩化コバルト	1.0%pet.
5	塩化アルミニウム	2.0%aq..	15	硫酸ニッケル	2.5%pet.
6	塩化パラジウム	1.0%aq.	16	塩化鉛	2.0%pet.
7	塩化白金酸	0.5%aq.	17	塩化パラジウム	1.0%pet.
8	硫酸銅	1.0%aq.	18	Ammonium tetrachloroplatinate	0.25%pet.
9	塩化第二水銀	0.005%aq.	19	臭化銀	2.0%pet.
10	塩化亜鉛	0.5%pet.	20	チタン粉末	20%pet

pet.:petrolatum　aq.:aqueuos solution

る。

①試験試料：日本接触皮膚炎学会のJapanese standard allergens（以下JSAと略す；表2）は，金属アレルギーのみならず，他の思いがけない接触アレルギーの原因検索としても有用である。また特に，金属アレルギーが疑われる場合は同時に当科では，金属シリーズとして表3に示す試料もパッチテストしている。

②貼布方法と判定：皮疹のない背部に，フィンチャンバー®，スカンポールテープ®を用いて，48時間閉鎖貼布する。ユニット除去1時間後（D2），24時間後(D3)，5日後(D7)にInternational Contact Dermatitis Research Group(ICDRG)基準に従い判定する。金属アレルゲンの反応には，刺激反応や遅発反応があるので，D7の判定は必要不可欠である。

③JSAの陽性率

足立らの報告によると[7]，JSA24種についての1994年度の日本接触皮膚炎学会の集計では，上位10位中4種の金属アレルゲンが含まれていた。当科の1994年から2000年まで

表4　Japanese standard allergens の陽性率(1994～2000)

	Total(n=1302)		%	Female (n=979)		%	Male (n=323)		%
1	Cobalt chloride	208	16	Cobalt chloride	152	15.5	Cobalt chloride	56	17.3
2	Nickel sulhfate	164	12.6	Nickel sulhfate	142	14.5*	Paraphenylene-diamine	50	15.5
3	Gold sodium thiosulfate	142	10.9	Gold sodium thiosulfate	106	10.8*	Paraphenylened iamine	32	9.9
4	Potassium dichlomate	129	9.9	Potassium dichlomate	79	8.1	Urushiol	32	9.9
5	Fradiomicin sulfate	83	6.4	Fradiomicin sulfate	60	6.1	Timerosal	26	8
6	Paraphenylenedi -amine	78	6	Paraphenylene-diamine	46	4.7	Fradiomicin sulfate	23	7.1
7	Urushiol Ammoniated	72	5.5	Urushiol	40	4.1	Nickel sulhfate Ammoniated	22	6.8
8	mercuric chloride	58	4.6	Mercuric chloride Ammoniated	34	3.5	mercuric chloride	18	5.6
9	Mercuric chloride	48	3.7	mercuric chloride	32	3.3	Gold sodium thiosulfate	14	4.3
10	Timerosal	44	3.4	Timerosal	26	2.7	Mercuric chloride	14	4.3

*：$p < 0.05$

表5　金属とその感作源

金属	感作源
コバルト	メッキ製品（ニッケルメッキされたものにはほとんど入っている）歯科金属、セメント、染毛剤、印刷インキ、ビタミンB12(メチコバール)、接着剤、色素、絵の具、クレオン、陶磁器、エナメル、粘土、ポリエステル系プラスチック
ニッケル	ニッケル合金製品、ニッケル硬貨（50円、100円）、歯科金属、ステンレス製品（台所用品）、ステンレス製医療機器（プレート、ペースメーカー、人工弁、人工骨、注射針など）、陶磁器、媒染剤、磁石、塗料、ガラス、エナメル、食品（缶詰製品、牡蠣、緑黄色野菜、ココア、チョコレート、そば、海苔、オートミール、紅茶など）
クロム	皮革製品（革靴、ベルト、かばんなど）、セメント、インク（ボールペン、印刷インクなど）、クロムメッキ製品、歯科金属、マッチの軸木、黄色ペンキ、媒染剤、緑色衣料、緑色ネル、ゴム、ガラス、トタン、防錆剤、毛皮処理剤など
金チオ硫酸ナトリウム	金装飾品（ピアス、ネックレス、イヤリング、時計など）、歯科金属、金合金製品、貴金属回収作業、医薬品（シオゾール、リドーラ、オーラノフィンなど）、金粉、金箔、金貨、メダル、工業用品など
チメロサール	殺菌剤、抗真菌剤、防腐剤（医薬品、化粧品、ワクチンなど）
塩化水銀アミド	歯科金属、農薬、種子消毒、防腐剤、殺菌剤、医薬品（止血剤、消毒薬など）、体温計、朱肉、朱色など
塩化第二水銀	乾電池、染料、防腐剤、殺菌剤、殺虫剤、木材保存剤、帽子製造、写真、印刷、金属エッチング、皮なめし、医薬品（消毒薬、防腐剤、髪の強壮剤、頭皮の治療薬など）

の7年間に施行した1302例のJSA27種と金チオ硫酸ナトリウムのパッチテスト結果を表4に示したが，上位10位内に金属アレルゲンが7種も含まれ，いかに金属アレルゲンが日常生活に密着したアレルゲンであるかが伺える。またニッケルと金チオ硫酸ナトリウムは，統計学的にも女性に多いアレルゲンで有意差があった[8]。JSAのパッチテストで陽性を示した場合に，当科で患者さんに渡す説明書の中から金属アレルゲンのみを抜粋したものを表5に示す。

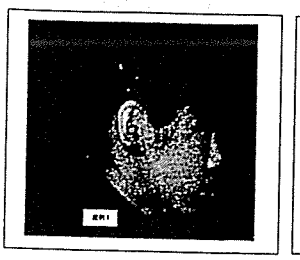

写真1

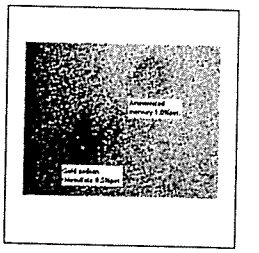

写真2

写真1　24歳，女性。ピアスによる皮膚炎，耳朶の紅斑、腫脹をきたしている。
写真2　パッチテスト1週間後（D7）判定で，Ammoniated mercury 1.0%pet., Gold sodium thiosulfate 0.5%pet に強陽性を示した。

4. 治療

一旦金属アレルギーが成立すると，減感作はできないので，パッチテストで陽性を示した金属アレルゲンを含有している物質との接触を避けることが治療の基本となる。そのうえで，対症療法として，症状に応じてステロイド外用薬や抗アレルギー薬の内服を行っていく。インタール®や金属キレート作用のあるミノマイシン®が有効なこともある[9]。

5. 金属接触アレルギーの発症予防と対策方法

金属アレルギーの発症予防には，夏季などの金属がイオン化を起こし，溶出しやすい環境下で発汗の多い部位への金属の長時間接触を避ける。皮膚のバリア障害が起きている部位への金属の接触を避けるなどの指導を徹底して行うべきである。やむをえず金属製品の使用を避けられない場合は，①金属のイオン化が起きないようにする，②感作性の高い金属を使用しないようにする，③金属製品に金属成分標示を付ける，④着脱可能なものにする，⑤短時間の使用にする，⑥汗をかかないようにする，⑦メタルコート剤を使用する，⑧純チタン製，⑨非金属，⑩合成皮革などの代替品を利用する，⑪手袋着用や衣服の上から使用するなど直接皮膚に金属製品を使用しないようにするなどの感作源となる金属アレルゲンの暴露を最小限にする防御方法の指導をする必要がある。

全身型の接触皮膚炎の場合は，パッチテスト陽性を示した金属アレルゲンが多量に含有されている食品の摂取を制限し，パッチテスト陽性の歯科金属の除去を考慮する。

一旦成立した金属の接触アレルギーは，減感作はできない。金属の接触感作の予防をできる限りしていかなければならない。

6. 症例供覧

症例1：24歳，女性。
既往歴：アトピー性皮膚炎。
家族歴：特記すべきことなし。
現病歴：初診の約1年4カ月前に，18Kピアスを装着した。初診の2週間前から，両耳朶

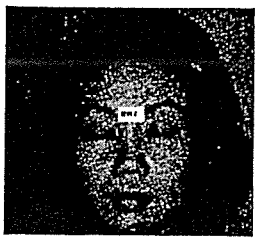

写真3

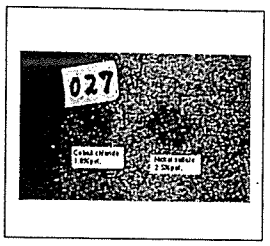

写真4

写真3　2824歳, 女性。両上眼瞼の浮腫性紅斑。
写真4　パッチテスト1週間後（D7）判定で, Cobalt chloride 1.0%pet., Nickel sulfate 2.5%pet. に強陽性を示した。

の紅斑，腫脹をきたし，疼痛を伴うようになったために当科を受診した。
初診時現症：両耳朶の紅斑，腫脹をきたし，軽度の疼痛とそう痒感を伴っていた（写真1）。
パッチテスト結果：Ammoniated mercury 1.0%pet.，Gold sodium thiosulfate 0.5%pet.に強陽性を示した（写真2）。
治療：very strongクラスのステロイド外用薬と抗アレルギー薬内服を約2カ月継続し略治した。

症例2：28歳，女性。
既往歴：蕁麻疹。
家族歴：特記すべきことなし。
現病歴：初診の約1年前より，ステンレス製のビューラーを使用していた。初診の6日前にステンレス製の毛抜きで眉の手入れをした。その翌日から，そう痒感が出現し，徐々に増強するために当科を受診した。
初診時現症：両上眼瞼に浮腫性紅斑が出現し，そう痒感を伴っていた（写真3）。
パッチテスト結果：Cobalt chloride 1.0%pet., Nickel sulfate 2.5%pet.に強陽性を示した（写真4）。
治療と経過：ステンレス製のビューラー，毛抜きを中止し，プラスチック性に変更し，very weekクラスの眼軟膏を外用し抗アレルギー薬内服を約1カ月継続し略治した。

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<話題あれこれ>

第35回　日本免疫学会総会・学術集会

日本免疫学会では下記日程にて第35回総会・学術集会を開催いたします。
会　期：2005年12月13日（水）～15日（木）　会　場：パシフィコ横浜
大会長：高津聖志（東京大学医科学研究感染・免疫部門免疫調節分野）
プログラム：
臓器特異的自己免疫疾患：発症と制御の基礎／臓器特異的自己免疫疾患：ヒト病態解明の戦略／他
学会事務局：〒101-0061　東京都千代田区三崎町3-6-2 原島三崎町ビル1F　日本免疫学会 事務局
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皮膚テスト（プリックテスト・皮内テスト・パッチテスト）

矢上晶子、松永佳世子（藤田保健衛生大学医学部皮膚科学講座）

はじめに

日常診療では、アトピー性皮膚炎、花粉症、食物や薬剤アレルギー、かぶれなどのさまざまなアレルギー疾患に遭遇する。これらの疾患に対してアレルゲンを確定するためには詳しい問診を行い、必要な検査方法を選択し、結果の解釈を正しく行うことが大切である。得られた結果より適切な生活指導を行えば症状を改善することができる。

皮膚アレルギー検査には大きく分けて、即時型（Ⅰ型）アレルギーを検索するプリックテスト、スクラッチテストおよび皮内テストと遅延型（Ⅳ型）アレルギーを検索するパッチテストが挙げられる。

本稿では、即時型および遅延型アレルギーに用いられるこれらの検査を具体的に解説したい。

即時型アレルギーの検査方法：プリックテスト、スクラッチテスト、皮内テスト

プリックテスト、スクラッチテスト、皮内テストは特異IgE抗体の関与した即時型アレルギーの診断に用いる*in vivo*検査法である[1,2]。これらの検査は患者の前腕屈側を用いて一度に複数のアレルゲンを用いて確認することができる。

1　プリックテスト

食物アレルギー、花粉症に伴う口腔過敏症状、ラテックスアレルギーおよびラテックス－フルーツ症候群、薬剤アレルギー、アトピー性皮膚炎、花粉症などに対して実施する。

プリックテストの利点は水溶性抗原に高い感度を示し、検査に伴うアナフィラキシーショックの危険性が皮内テストに比べて低いことや、負荷テストを行うことなく果物、魚介類、小麦、天然ゴムラテックス製品などのアレルゲンを検査することができることにある[3-5]。

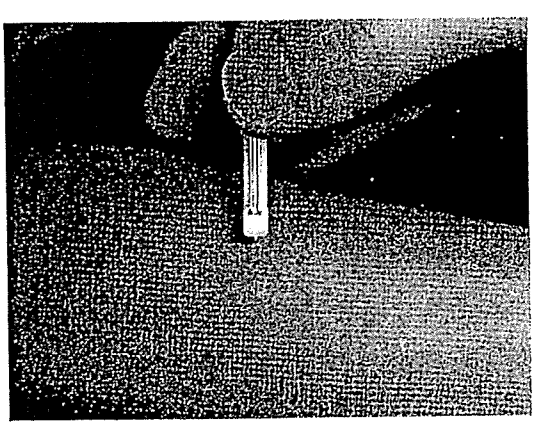
1）プリックテストの反応のメカニズム

①皮膚表層に点状に傷をつけて、ごく少量のアレルゲンを入れる（図1）。②皮膚表層に入ったアレルゲンが浸透し、肥満細胞の高親和性IgEレセプター（$Fc\varepsilon RI$）に結合したIgE抗体に架橋されると脱顆粒を起こしヒスタミンが放出される。③15分以内にwheal and flare reactionが生じ、膨疹の大きさで判定する（図2）。

2）検査に必要な器具

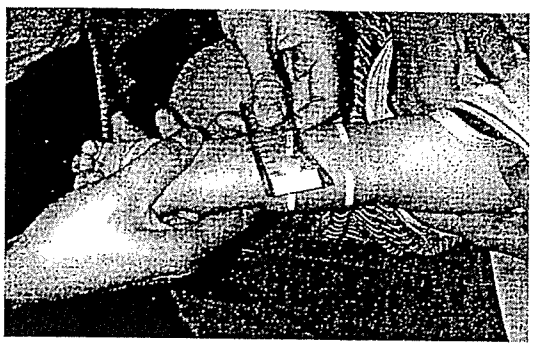
プリックランセット[EWO CARE AB, Sweden, 日本では（株）ヤヨイ、Tel：03-3813-5816で購入できる]、滅菌生理食塩水（以下、生食と略す）、二塩酸ヒスタミン

図1　プリックテスト



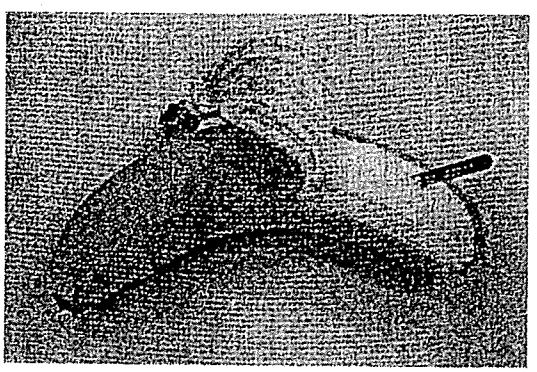
プリックランセットで皮膚表面に軽く傷をつける

図2：プリックテストの判定



15分後に膨疹の直径mm（最長径とその中点に垂直な径の平均値）を測定する

図3：新鮮な野菜や果物を用いたprick to prick test



(10mg/mL)、アレルゲン(果物、卵など)、消毒綿(イソプロパノールや70%エタノール)、タイマー、判定板(紅斑から膨疹を区別するためにはツベルクリン反応判定用硝子板がよい)、ティッシュペーパー、アレルゲンをプリックする部位に貼付するシールが必要である。

3)手技

①テストは前腕屈側で行う。各アレルゲンの間隔は少なくとも3cmおき、肘から3cm、手首から5cm離す。②テストする部位に抗原名を記入したシールを貼付する。③アレルゲンを一滴置く。④プリックランセットで皮面に対して90°の角度でアレルゲンを貫いて静かに1度刺す。ランセットは抗原ごとに消毒綿で拭き、1人の患者に対し1本を使用する。なお、消毒綿に対してアレルギーや刺激反応をもつ患者に対しては蒸留水を用いている。⑤アレルゲンをティッシュペーパーで拭き取る。⑥新鮮な材料(果物や野菜)を検査に用いる場合はprick-prick methodで行う(図3)。新鮮な果物などに刺したプリックランセットをそのまま皮膚に刺す。

4)プリックテストの判定

15分後に膨疹の直径mm(最長径とその中点に垂直な径の平均値)を測定し、紅斑は判定対象としない。対照液は陽性コントロールとして二塩酸ヒスタミン：10mg/mL(和光純薬)(1% 二塩酸ヒスタミンは前腕屈側では3～8mmの膨疹をつくる)、陰性コントロールとして生食を用いる。ヒスタミンの2倍を4＋、同等を3＋、2分の1を2＋、2分の1より小さく、生食より大きいものを1＋、生食と同等を(－)と判定する。判定結果が2＋以上を陽性とする。

2　スクラッチテスト

スクラッチテストは、プリックテストが陰性の場合に行う。患者の前腕屈側にペンでマーキングした後、プリックランセットないしは細い注射針(23G)で皮膚表面を出血しない程度に線状の傷(約5mm)をつける。判定はスクラッチした線と垂直な幅の膨疹径を測定し、プリックテストと同様に陽性・陰性コントロールと比較している。この検査は吸収される抗原量が増えるため、プリックテストに比べ感度は上がる。しかしながら、傷をつける程度によっては刺激反応が生じやすくなるため、結果の評価や検査を行う者の技術が必要となる。

3　皮内テスト

アトピー性皮膚炎、花粉症、気管支喘息などのアトピー疾患におけるアレルゲンには、市販されている皮内テスト用アレルゲンを用いる。市販されていないアレルゲンを独自に作製し使用すると、皮膚組織への刺激や傷害を与えることがあるため注意を要する。具体的には、0.01mLの目盛りがついている注射器に26Gの皮内針をつけたものを皮内テスト用注射器として用い、アレルゲン液0.02mLを前腕屈側の皮内に注射する。対照液として生食を用いる。判定は15分後に膨疹と発赤の長径と短径を測定し、膨疹(長径×短径)/発赤(長径×短径)として記載し、表1の皮内テストの判定基準に従って判定する。即時型アレルギーの場合でも、数時間から翌日

にかけて遅発性の紅斑反応が生じることがあるため、24時間後にも判定を行うことが望ましい。

最近の話題として、抗生物質アナフィラキシーに対する予知テストとしての即時型皮内テストの是非が問われている[6]。厚生労働省は2004年10月28日発行の「医薬品・医療用具等安全性情報206号」で、注射用抗生物質製剤等によるショック等に対する安全対策において皮内テストの廃止を報じた。それは、皮内テストの実施によりアナフィラキシーを引き起こした症例がある一方で、皮内テストが陰性であった抗生物質の全身投与によりアナフィラキシーショックを引き起こす症例が存在したためである。今後の対策として、①抗生物質製剤の投与前に問診により患者のアレルギー歴の確認をする。②ショック等に対する救急処置のとれる準備をしておく。③投与開始から終了後まで十分な観察を行う。④病歴から即時型薬剤アレルギーが疑われる患者に対してはプリックテストを行う、などを挙げている。

上述したいずれの検査も、アナフィラキシーショックを誘発する可能性があるため、症例によっては点滴ルートを確保し、緊急時にはエピネフリンの筋肉注射などの救命処置を行えるよう準備を行っておくとよい。

遅延型アレルギーの検査方法：パッチテスト

かぶれ、すなわち遅延型アレルギー(Ⅳ型アレルギー)であるアレルギー性接触皮膚炎に対してはパッチテストを行う[7]。これは、患者の皮膚にアレルゲンを塗布または貼布することによって原因アレルゲンを明らかにする検査である。まず、十分な問診を行い、患者の皮膚炎の原因となったアレルゲンを選択することが大切である。そして、原因と考えられるアレルゲンを適切な方法で貼布し、標準化された判定基準に沿って判定することにより正しい結果を得ることができる。

表1 即時型皮内テストの判定

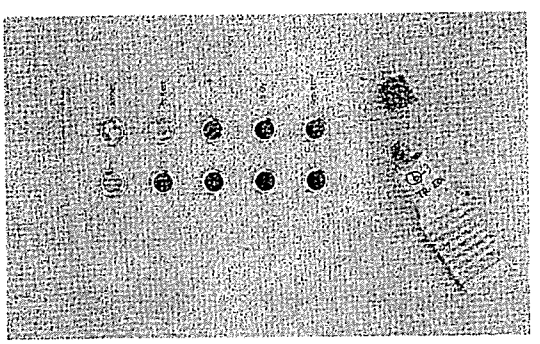
判定	反応
−	発赤径　10mm以下
±	発赤径　11～20mm
＋	発赤径　21mm以上、膨疹径9mm以下
＋＋	発赤径21～40mm、膨疹径10～14mm
＋＋＋	発赤径41mm以上、膨疹径15mm以上および明らかな偽足を示すもの

1) パッチテストの方法

単純パッチテスト(closed patch test)

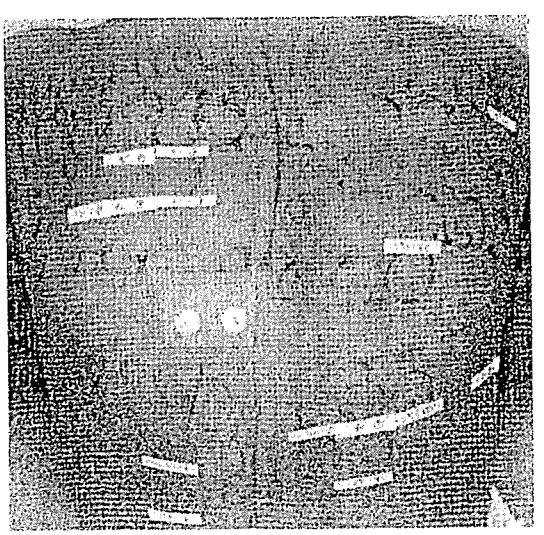
背部(傍脊椎部)の皮膚病変のない皮膚にアレルゲンを閉鎖貼布する。具体的にはパッチテストユニットScanpor tape (Norgesplaster, Norway：大正製薬)上にFinn Chamber (Epitest, Finland)がついているユニット(図4)やパッチテスト用絆創膏(鳥居薬品、東京)を用いてアレルゲンを閉鎖貼布する。そして、48時間後にアレ

図4 パッチテストユニット



Scanpor tape (Norgesplaster, Norway：大正製薬)上にFinn Chamber (Epitest, Finland)がついているユニットを用いている

図5 パッチテストの判定(48時間後判定)



ルゲンを除去し、絆創膏による圧迫刺激の影響がなくなる約1時間後に48時間後判定を行う(図5)。以降、72時間後、さらに1週間後判定を行う。

オープンテスト

染毛剤、パーマ液、脱毛クリームや揮発性の製品は原液のままオープンテストを行う。すなわち、製品(試料)を直径20mmの円に単純塗布し、20～30分後に膨疹反応の有無を判定し、その後、48時間、72時間後に判定する。

2)アレルゲンの入手方法

代表的なパッチテスト用アレルゲンは数社から市販されている。多くのアレルゲンを取りそろえているBrial(Germany)のアレルゲンは海外技術交易(TEL：03-3275-3461)より入手できる。また、国産では、鳥居薬品のアレルゲンが入手可能である。

入手できないアレルゲン(製品中の成分など)は"Contact Dermatitis"[8]や各アレルゲンの希釈方法や濃度が記載されている参考文献を用いてそれらを確認し作製する。しかしながら安全が確認できないものについては感作させたり、皮膚に傷害を与える可能性があるため貼布しないほうがよい。

3)各製品(アレルゲン)の希釈および貼布方法

以下に、各製品の調整方法を記載する。

化粧品：口紅、化粧水など皮膚に直接塗布する製品はそのまま貼布する。シャンプー、せっけん、洗顔料などの洗浄剤は1% 水溶液を作製して貼布する。前述した染毛剤などは原液でオープンテストを行う。**外用剤**：ゲル製剤は刺激反応があるため製品を用いてオープンテストを実施し、湿布類はそのまま貼布する。**点眼液**：製品の濃度で貼布する。しかし、眼瞼結膜に比べると背部の皮膚は経皮吸収が低いので、通常のパッチテストと皮膚をスクラッチした部分に製剤を貼布するスクラッチパッチテストの両方を行う。**金属**：ヤスリで削りワセリンに混ぜて貼布する。**食品類**：そのまま貼布する。**植物**：花びら、葉、茎に分けてすり潰して貼布する。しかし、さくら草は強い感作性をもつのですり潰した後に水で10倍に希釈して貼布する。刺激性のある植物は10% 水溶液あるいはエタノール、アセトンで抽出液を作る。**農薬**：ワセリン、水ないしは親水ワセリンに使用濃度、10倍希釈濃度で混ぜて貼布する。**消毒薬**：製品を貼布する。刺激性のあるものは使用濃度でのオープンテストを行う。使用テストも有用である。

4)パッチテストを受ける患者への注意点

以下の事柄を、アレルゲンを貼布する前に患者へ説明し同意を得た上でパッチテストを開始するとよい。①パッチテストを行うことで感作を起こす可能性があること。②患者の湿疹病変を再燃させることがあること。③反応が強く、アレルゲンの貼布部位に水疱などが出現することがあること。④パッチテスト後に色素沈着や色素脱失をきたすことがあること。

5)判定基準

判定基準には、アレルギー反応の判定に適している国際接触皮膚炎研究班(International Contact Dermatitis Research Group; ICDRG)基準[9]と刺激反応を含めて判定することができる本邦基準[10]がある(表2)。

表2 パッチテストの判定基準

本邦基準	反応	ICDRG基準	反応
刺激反応も含めて判定できる		アレルギーの判定に適している	
−	反応なし	−	反応なし
±	軽度の紅斑	+?	紅斑のみ
+	紅斑	+	紅斑＋浸潤、丘疹
++	紅斑＋浮腫、丘疹	++	紅斑＋浸潤＋丘疹＋小水疱
+++	紅斑＋浮腫＋丘疹＋小水疱	+++	大水疱
++++	大水疱	IR	刺激反応
		NT	施行せず

ICDRG：国際接触皮膚炎研究班

6) パッチテスト反応の解釈と注意点

陽性反応が得られた場合、その結果を正しく解釈するためには、①接触または使用歴を再度問診し、今回の皮膚炎の原因か、増悪因子かを明らかにする。②今回接触した物質でなければ過去の皮膚炎の既往を十分に問診し、以前の皮膚炎の原因か、増悪因子かを明らかにする。③さらに、これまでの皮膚炎とは関係のない場合、交叉反応である可能性を考慮するなどを検討する。一方、パッチテストが陰性の場合は、アレルゲンを正しい濃度や適切な方法で貼布したかなどを検証する。特に強い陽性反応を呈したアレルゲンの近傍では非特異的に陽性反応が惹起されることがあり、これをexcited skin syndrome[11]とよぶ。この反応は多感作や交叉反応の判定と識別することが困難であることが多い。

7) 診断

アレルギー性接触皮膚炎を診断する際には上述のごとく検査を行い、発症経過や臨床症状とパッチテストの結果を考慮して診断を行う。

8) 患者への説明

患者へは、1週間後判定時にパッチテストの結果を説明する。交叉反応するアレルゲンがある場合には、これらも説明し接触しないように注意する。また、陽性反応を呈したアレルゲンが今回の皮膚炎と関連があったかを再度確認する。持参品については陰性の製品を報告し、今後の生活に役立てていただく。持参品が陽性の場合は、その製品の成分による再パッチテストが必要となることを説明し、準備を進める。成分パッチテストを行うことで、患者は同様の成分を含んだ製品も避けることが可能となる。しかし、成分(アレルゲン)の調製は専門書で確認しなければならないのでパッチテストを専門とするアレルギー外来に紹介することを勧める。また、代替品がある製品についてはそれらを紹介し、接触を回避するための防御対策法についても指導する。もし、職業性の接触皮膚炎の場合には、患者の職場にパッチテストの結果を報告し職場の異動を勧める。接触皮膚炎についてより多くの情報を入手したい方は日本接触皮膚炎学会のホームページを参考にしていただきたい(http://www.fujita-hu.ac.jp/JSCD/)。

おわりに

皮膚アレルギーは皮膚科医に限らず、日常診療でよく遭遇する疾患の1つである。皮疹の形態や経過、詳細な問診から適切な検査方法を選択し原因をつきとめれば予防することができるため、患者のQOLに非常に役立つ検査と思われる。しかしながら、検査方法やその解釈に対する幅広い知識と確実な手技、判定力が必要であることを筆者は日々痛感している。今後もより良い検査方法の確立を目指したい。

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ORIGINAL ARTICLE

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Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during in vitro culture in comparison with cancer cells

Abstract We investigated the expression levels of several genes related to cell proliferation in human mesenchymal stem cells (hMSCs) during in vitro culture for use in clinical applications. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. The proliferation rate of hMSCs gradually decreased and marked changes in hMSC morphology were not observed in 3 months of in vitro culture. The mRNA expressions of TGFβ1, TGFβ2, and TGFβ receptor type I (TGFβRI) in hMSCs increased with the length of cell culture. There had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage from the primary culture (at about 3 months). The mRNA expression of Smad3 increased, but those of c-myc and nucleostemin decreased with the length of hMSC in vitro culture. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells. In conclusion, hMSCs derived from bone marrow seldom underwent spontaneous transformation during 1–2 months of in vitro culture for use in clinical applications. In hMSCs as well as in epithelial cells, growth might be controlled by the TGFβ family signaling.

Key words Stem cells · Cell proliferation · TGFβ signaling · TGFβ receptors

Introduction

Several recent studies demonstrated the potential of bioengineering using somatic stem cells in regenerative medicine.[1,2] Bone marrow includes both mesenchymal and hematopoietic stem cells. Adult human mesenchymal stem cells (hMSCs) derived from bone marrow have the pluripotency to differentiate into cells of mesodermal origin, e.g., bone, cartilage, adipose, and muscle cells.[1–5] Moreover hMSCs also have the capacity to differentiate into myocytes,[6,7] hepatocytes,[1,8] and neural cells.[3] In addition, because they are comparatively easy to expand ex vivo, hMSs have many potential clinical applications, not only in the field of orthopedic surgery but also for the treatment of cardiac infarction, cirrhosis, and diabetes. On the other hand, stem cells possess a self-renewal capability similar to that of cancer cells.[9] Recently Rubio et al.[10] reported spontaneous transformation of human adult stem cells derived from adipose tissue in long-term (4–5 months) in vitro culture. In practice, if hMSCs are to be used for clinical applications and tissue-engineered medical devices, they have to be expanded in vitro for about 1–2 months. The proliferation ability and the gene expression profile of hMSCs, however, might change during in vitro culture. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. TGFβ is a multifunctional protein that regulates cellular proliferation, differentiation, apoptosis, development, extracellular matrix formation, immunosuppression, and tumorigenesis. In humans, three TGFβ isomers have been identified: β1, β2, and β3. TGFβ signals through three high-affinity cell surface receptors: TGFβ type I (TGFβRI), type II (TGFβRII), and type III (TGFβRIII) receptors. TGFβRI and TGFβRII are serine-tyrosine kinases. TGFβRIII is known to be a betaglycan.[11] TGFβs are first bound to TGFβRII and TGFβRIII.[12] It has been considered that TGFβRIII regulates access to TGFβRII,[12–14] and then TGFβ signal transduction in the cellular pathway is started through stimulation of TGFβRI by TGFβRII. After that, activated TGFβRI phosphorylates Smad2 or Smad3, which are receptor-regulated Smads (R-Smad) activated by TGFβ and activin.[15,16] After Smad4, which is a common mediator Smad (C-Smad), is connected to phosphorylated R-Smads, the complex is transported to the cell nucleus and influences the transcription activity of TGFβ-dependent genes.[15,16] c-myc, which is one of the

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TGFβ-dependent genes, is regarded as an oncogene and regulates cellular proliferation. In the present study, therefore, we investigated whether the gene expression levels of three TGFβ isomers (TGFβ1, TGFβ2, and TGFβ3) and their receptors (TGFβRI, TGFβRII, and TGFβRIII), Smad3 and c-myc were changed in hMSCs during in vitro culture.

Wnt-8B is related to cell self-renewal and tumorigenesis,[9] and Wnt proteins can act as stem cell growth factors.[17] Wnt signaling activates the genes that promote proliferation (c-myc and others) by accumulating β-catenin in some kinds of stem cells and cancer cells.[9] Nucleostemin is involved in proliferation in both stem cells and cancer cells.[18] Therefore we also investigated the gene expression levels of Wnt-8B and nucleostemin in hMSCs.

In addition to investigating the expression of these genes r ing to cellular proliferation in hMSCs during in vitro culture, we compared them with those in two kinds of cancer cell lines, HeLa S3 (a human cervical cancer cell line) and HepG2 (a human hepatoma cell line).

Materials and methods

Cell culture. Human mesenchymal stem cells (hMSCs) derived from bone marrow were purchased from Cambrex Bio Science (Walkersville, MD, USA). Their donor was an African American woman aged 19 years. The cells that we obtained from Cambrex Bio Science were second-passage cells. The hMSCs were cultured in mesenchymal stem cell basal medium (MSCBM; Cambrex Bio Science) supplemented with mesenchymal cell growth supplement (MCGS; Cambrex Bio Science), L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C under a 5% CO_2 atmosphere. The cells were seeded at a density of 6000 cells/cm^2 ; were subcultured when they were just subconfluent (approximately 90% confluent) up to the 10th passage, corresponding to the 12th passage from when the hMSCs were collected from the donor. The human cervical carcinoma cell line HeLa S3 (JCRB Cell Bank, Osaka, Japan) was cultured using Ham's F-12 culture medium (Dainippon Pharmaceutical, Osaka, Japan) containing 10% fetal bovine serum (FBS) (Intergen, Purchase, NY, USA) and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The human hepatoma cell line HepG2 (Riken Bioresource Center, Tsukuba, Japan) was cultured using minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1 mM nonessential amino acids (NEAA) (Invitrogen), 10% FBS (Intergen), and 100 U/ml penicillin-streptomycin (Invitrogen).

Preparation of total RNA. Because the purchased hMSCs had been expanded in the manufacturing process as described above, we express the 1st passage of the hMSCs in this study as the 3rd from the primary culture. For quantitative real time-polymerase chain reaction (RT-PCR), total RNA was extracted from hMSC cultures during the 3rd, 5th, 7th, and 12th passages from the donor with Isogen (Nippon Gene, Toyama, Japan). Total RNA was also extracted from HeLa S3 and HepG2 cells once only with Isogen (Nippon Gene).

Quantitative RT-PCR. RNA was then reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Basel, Switzerland). Primers and annealing temperatures for the c-myc oncogene, nucleostemin, Wnt-8B, transforming growth factor (TGF)β3, and TGFβRIII are summarized in Table 1. Amplifications were carried out for 10 s at 95°C, for 15 s at each annealing temperature, and for 12 s at 72°C for 40 cycles. Amplifications of TGFβ1, TGFβ2, TGFβRI, TGFβRII, and Smad3, plus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, were performed using Light Cycler Primer Sets (Roche Diagnostics). PCR was performed in Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) in a Roche Light Cycler (software version 4.0).

Statistical analysis. All results are shown as means ± SD. The significance of the differences in mean values was evaluated by Student's *t* test.

Table 1. Primers and annealing temperatures used for real-time PCR

Gene name	GenBank accession number	Primer orientation	Nucleotide sequence	Starting sequence position	Size for the PCR amplicon(bp)	Annealing temp. (°C)
c-myc	V00568	Forward	5′- GCG AAC ACA CAA CGT C -3′	1626	315	50
		Reverse	5′- CAA GTT CAT AGG TGA TTG CT -3′	1940		
nucleostemin	X91940	Forward	5′- CCA TTC GGG TTG GAG TAA -3′	782	284	50
		Reverse	5′- CTG TCG AGC ATC AGC C -3′	1065		
Wnt-8B	NM_014366	Forward	5′- AGT GAC AAT GTG GGC T -3′	331	244	60
		Reverse	5′- CGT GGT ACT TCT CCT TCA G -3′	574		
TGFβ3	NM_003239	Forward	5′- AAA CAC CGA GTC GGA A -3′	535	284	60
		Reverse	5′- TGC CAC CGA TAT AGC G -3′	818		
TGFβRIII	NM_003243	Forward	5′- TCC CTA TCC CGC AAG C -3′	2369	189	60
		Reverse	5′- AGA TTA TCG AGG CGT CC -3′	2557		

PCR, polymerase chain reaction; TGFβ3, transfroming growth factor β3; TGFβRIII, TGFβ recepter type III

Results

The proliferation rate of hMSCs decreased with the length of in vitro culture (Fig. 1). The effects of the in vitro culture term on hMSC proliferation and the mRNA expressions of three TGFβ isomers (TGFβ1, β2, β3) and their receptors type I, II, and III (TGFβRI, RII, RIII) in hMSCs were investigated (Fig. 2). The mRNA expressions of TGFβ1, TGFβ2, and TGFβRI increased with the length of cell culture (Fig. 2A,B,D), but there had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage (at about 3 months) (Fig. 2C,E,F). In addition, the mRNA expression of Smad3, which is one of the R-Smads activated by TGFβ and activin, in hMSCs was investigated. The mRNA expression of Smad3 decreased in the 5th and 7th passages of hMSCs but increased in the 12th passage (Fig. 3). The mRNA expressions of c-myc in hMSCs were higher in the 5th and 7th passages than in the 3rd and 12th passages (Fig. 4A). The mRNA expressions of nucleostemin in hMSCs decreased with the length of cell culture (Fig. 4B).

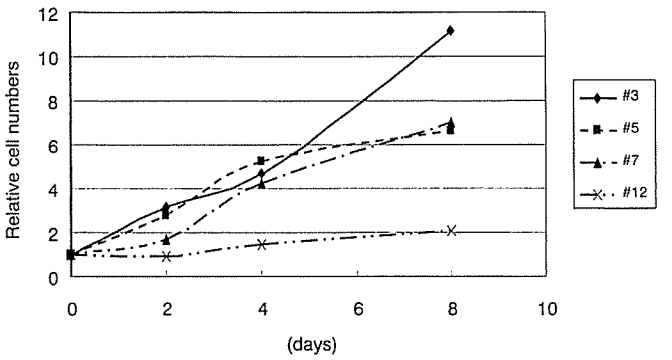


Fig. 1. Proliferation of human mesenchymal stem cells (hMSCs) in the 3rd, 5th, 7th, and 12th passages. hMSCs were seeded at 1.7×10^5 cells/F 60-mm dish (6000 cells/cm^2), and cells were counted after 2, 4, and 8 days. The initial cell number (0 days) is expressed as 1, and the other cell numbers (2, 4, and 8 days) are expressed relative to that of day 0. $n = 3$

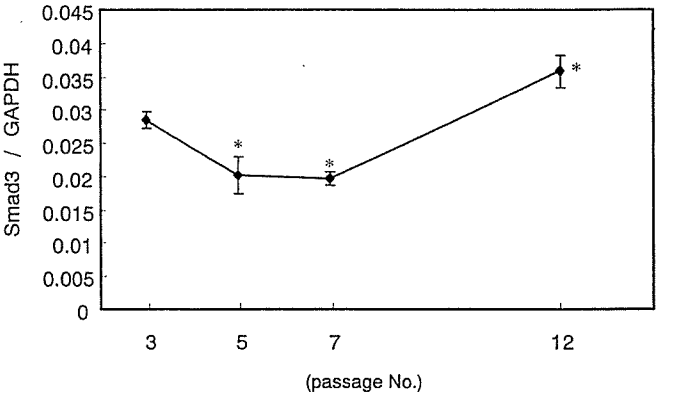
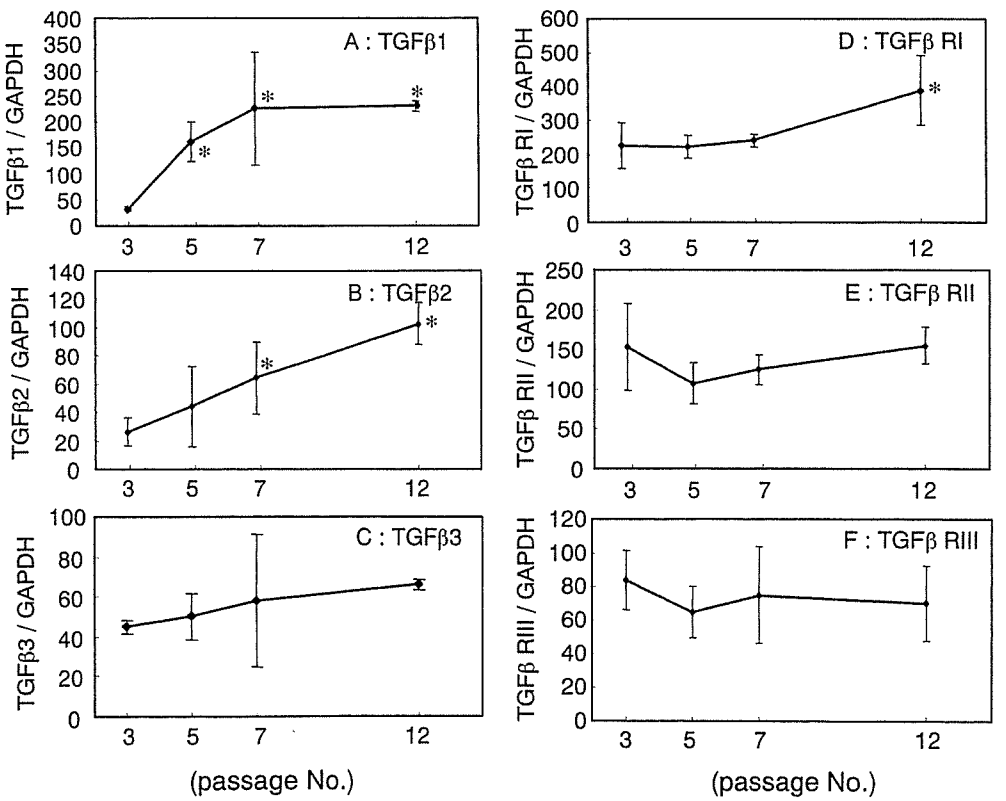


Fig. 3. Effect of in vitro culture length on mRNA expression of Smad3 in hMSCs. The expression of Smad3 relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages was investigated by quantitative RT-PCR. Mean values with SDs are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)

Fig. 2. Effect of in vitro culture length on mRNA expressions of transforming growth factor β1 (*TGFβI*) (**A**), TGFβ2 (**B**), TGFβ3 (**C**), TGFβ receptor type I (*TGFβRI*) (**D**), TGFβRII (**E**), and TGFβRIII (**F**) in hMSCs. Expressions of the four genes, relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative real time-polymerase chain reaction (RT-PCR). Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)



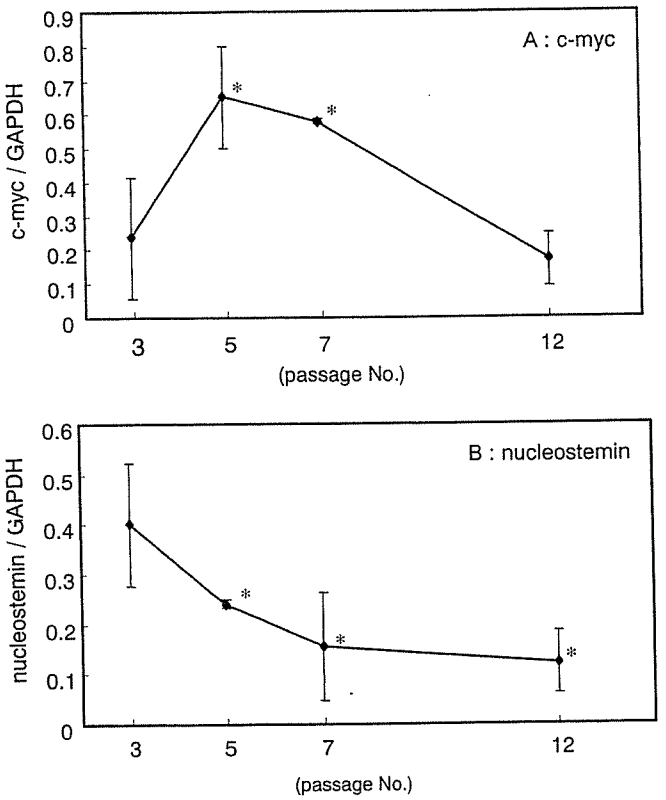


Fig. 4. Effect of in vitro culture length on the mRNA expressions of c-myc (**A**) and nucleostemin (**B**) in hMSCs. Expressions of the two genes relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative RT-PCR. Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)

The mRNA expressions of TGFβs and TGFβ receptors in hMSCs of the fifth passage were compared with those of two kinds of cancer cells (HeLa S3 and HepG2) (Fig. 5). TGFβ1 mRNA levels in hMSCs and HepG2 cells were significantly higher than those in HeLa S3 cells (Fig. 5A). The mRNA expressions of TGFβ2, TGFβ3, TGFβRI, and TGFβRII in hMSCs were significantly higher than those in the cancer cells (HeLa S3 and HepG2) (Fig. 5B,C,D,E). TGFβRIII mRNA expression in hMSCs was significantly higher than that in HeLa S3, but lower than that in HepG2 (Fig. 5F). The expressions of several genes affecting cellular proliferation in all three cells were also investigated. The mRNA expressions of c-myc oncogene and nucleostemin in the cancer cells (HeLa S3 and HepG2) were significantly higher than those in hMSCs (Fig. 6A and B). Wnt-8B mRNA was expressed in the cancer cells (HeLa S3 and HepG2), but not in hMSCs (Fig. 6C). Wnt-8B mRNA was not expressed in any passage numbers of hMSCs (data not shown).

Discussion

In this study, we investigated the changes of gene expression profiles during in vitro culture of hMSCs to evaluate their safety for use in clinical applications and tissue-engineered medical devices. First, the time dependency of the growth speed of hMSCs derived from bone marrow up to the 12th passage (at about 3 months) was investigated. The proliferation rate of hMSCs decreased by degrees during 3 months of in vitro culture (Fig. 1). No marked changes of hMSC morphology in 3 months of in vitro culture were

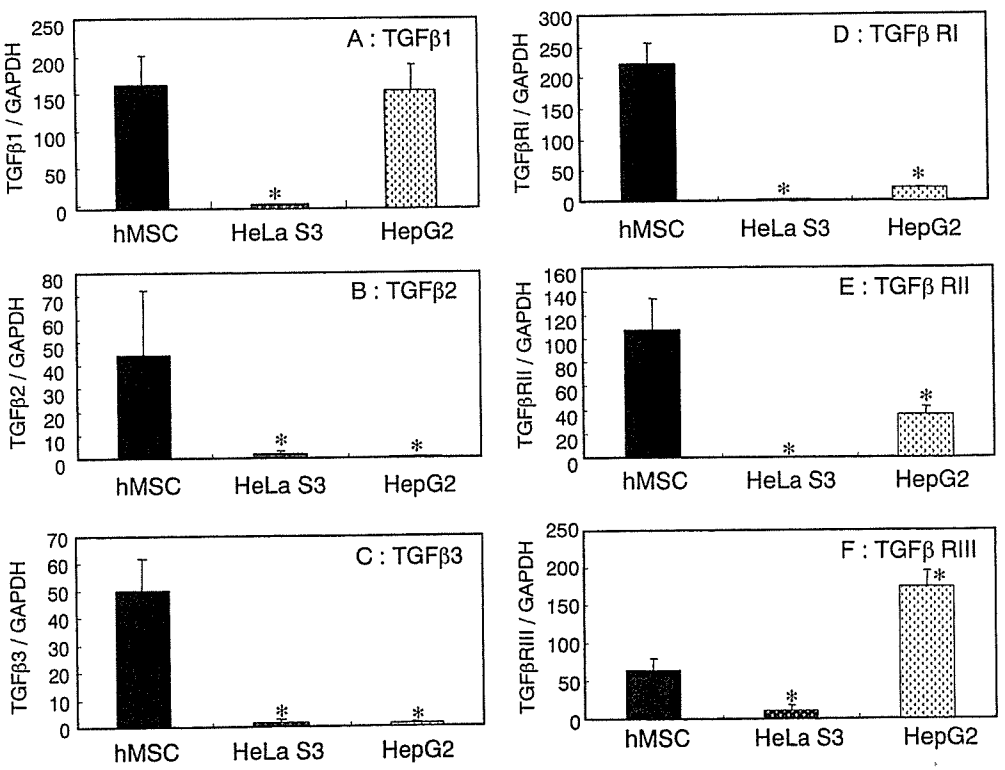


Fig. 5. mRNA expressions of TGFβ1 (**A**), TGFβ2 (**B**), TGFβ3 (**C**), TGFβRI (**D**), TGFβRII (**E**), and TGFβRIII (**F**) in hMSC, HeLa S3, and HepG2 cells. The expressions of the four genes relative to GAPDH in confluent cultures of hMSCs, HeLa S3, and HepG2 were investigated by quantitative RT-PCR. Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences from hMSCs (*$P < 0.05$)

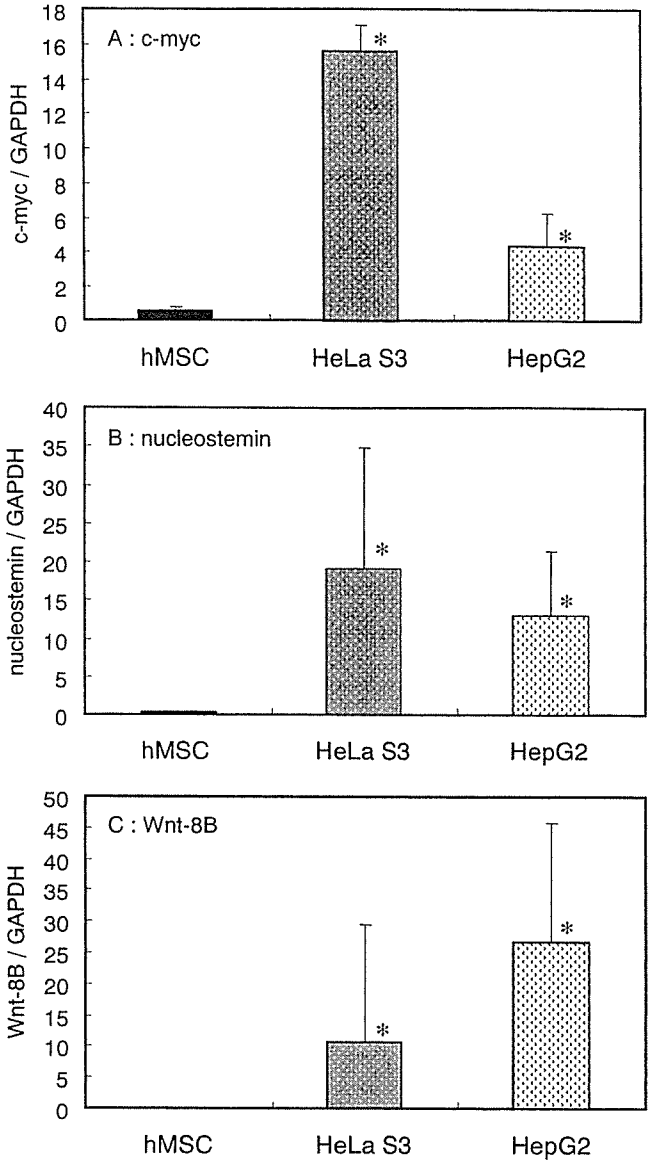


Fig. 6. mRNA expressions of c-myc (**A**), nucleostemin (**B**), and Wnt-8B (**C**) in hMSC, HeLa S3, and HepG2 cells. The expressions of the three genes relative to GAPDH in confluent cultures of hMSC, HeLa S3, and HepG2 cells were investigated by quantitative RT-PCR. Mean values with standard deviations from three independent experiments are presented. *Asterisks* denote statistically significant differences from hMSCs (*$P < 0.05$)

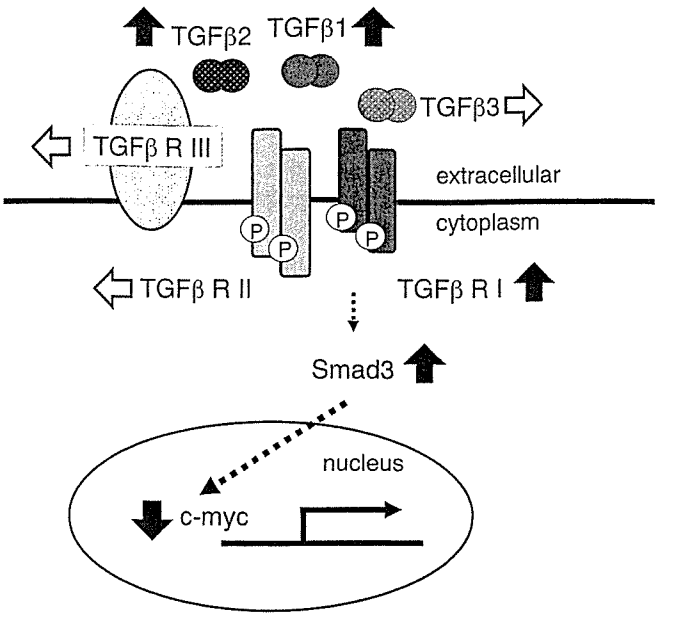


Fig. 7. Changes in the expressions of TGFβ signaling genes during hMSC in vitro culture for 3 months. The *dotted arrows* indicate the TGFβ signal pathway. *White arrows*, no changes; *black arrows*, up or down changes

observed. Several hMSCs derived from other donors' bone marrow did not undergo extraordinary proliferation either (data not shown). Adult stem cells have a self-renewal ability and undergo multilineage differentiation to maintain adult tissues.[9] In this study, however, hMSCs had more limited proliferative potential in in vitro culture. This phenomenon in hMSCs derived from bone marrow is the same result as that in hMSCs derived from adipose tissue reported by Rubio et al.[10] In addition, a decreasing cellular proliferation rate is often observed in several types of normal cells during in vitro culture. Consequently, these results suggest that hMSCs derived from bone marrow will seldom undergo spontaneous transformation during the 1–2 month period of in vitro culture necessary for use in clinical applications. But why does the proliferation of hMSCs decrease during in vitro culture? To focus on the proliferation mechanism of stem cells, we investigated whether the expressions of several genes related to cellular proliferation in hMSCs changed during in vitro culture. In the present study, we examined the expressions of TGFβs, their receptors, Smad3, c-myc, nucleostemin, and Wnt-8B. It has been proposed that the loss of TGFβRIII in renal cell carcinoma (RCC) is necessary for RCC carcinogenesis, and loss of TGFβRII leads to acquisition of the metastatic phenotype.[19] Therefore, the absence of changes in TGFβRII and TGFβRIII in hMSCs during in vitro culture might be important. The changes in mRNA expression levels during in vitro culture were different in each TGFβ isomer and receptor. TGFβ signal transduction in the cellular pathway is only possible through activation of TGFβRI. It was interesting that only TGFβRI mRNA expression increased with the length of cell culture among the three kinds of receptors (Fig. 2). The mRNA expressions of Smad3 increased (Fig. 3), but those of c-myc and nucleostemin decreased (Fig. 4) with the length of cell culture. We summarize the changes of TGFβ signaling gene expression during in vitro culture of hMSCs for 3 months in Fig. 7. TGFβ inhibits the growth of the many kinds of epithelial cells and hematopoietic, lymphoid, and endothelial cells.[20–23] In hMSCs as well as in the above-mentioned cells, hMSC growth might be controlled by TGFβ family signaling. As shown in Fig. 7, we hypothesized that the expressions of TGFβ1 and TGFβ2 in hMSCs increased during the period of in vitro culture, and then activated TGFβRI repressed the transcription of c-myc through Smad3; consequently, the cell cycle and cell growth might be arrested in hMSCs.

In addition, we compared the gene expression profiles of hMSCs with two kinds of cancer cell lines. One was HeLa S3 (a human cervical cancer cell line), which is markedly transformed, and the other was HepG2 (a human hepatoma cell line), which retains some hepatic functions. The mRNA expressions of TGFβs and their receptors in hMSCs were significantly higher than in the two types of cancer cells (HeLa S3 and HepG2) (Fig. 5). On the other hand, the mRNA expressions of c-myc and nucleostemin of the stem cells (hMSCs) were significantly lower than those of the two types of cancer cells (Fig. 6). Wnt signaling promotes self-renewal of hematopoietic, intestinal epithelial, and keratinocyte stem cells, among others;[9] however, Wnt-8B was not expressed in hMSCs derived from bone marrow (Fig. 6). These results suggest that expression of the genes that inhibit cellular proliferation and tumorigenesis were significantly higher and the genes that promote these processes were lower in hMSCs than in the cancer cells. Thus, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells.

Conclusion

In the present study, we confirmed that spontaneous transformation seldom occurred in hMSCs derived from bone marrow during 1–2 months of in vitro culture for use in clinical applications. In hMSCs, as in epithelial cells, growth might be controlled by TGFβ family signaling. During the period of in vitro culture of hMSCs, the expressions of TGFβ1 and TGFβ2 increased, and then activated TGFβRI repressed the transcription of c-myc through Smad3; consequently, the cell cycle and cell growth might have been arrested in hMSCs. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of the cancer cells.

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Effects of a biodegradable polymer synthesized with inorganic tin on the chondrogenesis of human articular chondrocytes

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Abstract: Recent study has shown that biodegradable polymers are attractive candidates for chondrocyte fixation and further transplantation in cartilage tissue engineering. Poly (glycolic acid) (PGA), a polymer of glycolic acid, is widely used in orthopedic applications as a biodegradable polymer. Organotin, lead, antimony, and zinc are catalysts commonly used in synthesizing PGA. Here, we investigated the biocompatibility of PGA, synthesized with and without inorganic tin as a catalyst in chondrogenesis of human articular chondrocytes in a micromass culture system. Significant enhancement of chondrocyte proliferation and expression of the collagen type II protein gene were observed in cultures treated with PGA synthesized with a tin catalyst. However, aggrecan gene expression was very similar to the control culture. Amount of collagen type II protein was also increased in the same group of cultured chondrocytes. In contrast, PGA without a catalyst caused overall inhibition of chondrogenesis. Despite several positive findings, extensive investigations are essential for the feasibility of this PGA(Sn) in future clinical practice. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 84–89, 2006

Key words: poly (glycolic acid); inorganic tin catalyst; human articular cartilage; chondrogenesis; micromass culture

INTRODUCTION

Different synthetic biodegradable polymers are currently gaining importance in the fields of biotechnology and tissue engineering. Recently, many studies have evaluated the potential of various natural bioabsorbable polymers such as collagen, [1,2] alginates, [3–5] fibrin, [6–8] and gelatin, [9] but synthetic biodegradable polymers in general offer advantages over natural materials. The primary advantages include the capacity to change the mechanical properties and degradation kinetics to suit various applications. Among the families of synthetic polymers, polyesters are used in a number of clinical applications.[10–12] Polyesters have also been used for development of tissue engineering applications, [13,14] particularly for bone tissue engineering.[15,12]

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The attraction of poly (glycolic acid) (PGA), one of the aliphatic polyesters, as a biodegradable polymer in medical applications is that its degradation product, glycolic acid, is a natural metabolite. Several studies have indicated that copolymers of glycolic acid caused promotion of nerve regeneration in a rat model, [16–18] and regeneration of an 80 mm nerve gap by an artificial nerve conduit made of PGA was also reported.[19] PGA can be synthesized using different catalysts. The common catalysts used include organotin, lead, antimony, and zinc. It was reported that inorganic and organic tin compounds present in the aqueous ecosystem have toxic effects and are capable of producing behavioral abnormalities in living organisms.[20,21] Organotin compounds are known to cause neurotoxicity, [22] cytotoxicity, [23] immunotoxicity, and genotoxicity [24] in human and other mammalian cells both *in vitro* and *in vivo*. Organotin compounds were also reported to decrease *in vitro* survival, proliferation, and differentiation of normal human B cells.[25] The dose effect of inorganic tin in rats suggests that the critical organ in inorganic tin toxicity is bone, [26] and disproportionate dwarfing syndrome, which severely affects the limbs but not the trunk, was observed in rats that had been injected with certain tin compounds.[27] As far as we know, no study yet has reported the chondrogenic

effects of PGA synthesized with and without an inorganic tin catalyst. In this study, the biocompatibility of PGA with and without a tin catalyst was investigated, using human articular chondrocytes (HAC) in a micromass culture system.

MATERIALS AND METHODS

Medium and polymers used for cell culture

Chondrocyte growth medium was obtained commercially from BioWhittaker (Walkersville, MD, USA). PGA synthesized with inorganic tin [PGA(Sn)] (M_w = 1500) and without a catalyst (PGA) (M_w = 1100) were custom-made (TAKI chemicals, Kakogawa, Japan) and dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical, St. Louis, MO, USA).

Cells and culture methods

Human articular chondrocytes (HAC) of the knee joint was commercially obtained from BioWhittaker. High-density micromass cultures were started by spotting 4×10^5 cells in 20 μL of medium onto Costar 24-well tissue culture microplates (Costar type 3526, Corning). After a 2 h attachment period at 37°C in a CO_2 incubator, culture medium (1 mL/well) was added to each well. Media were supplemented with DMSO (0.8 μL/mL), PGA, and PGA(Sn) (50 μg/mL). HAC cultured with DMSO was used as the control. The cultures were continued for 4 weeks with a medium change twice a week. At least four cultures were performed for each sample.

Cell proliferation study

Cell proliferation was quantitatively estimated by crystal violet (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[28] After the culture period, cells were fixed with 100% methanol at room temperature, followed by application of 0.1% crystal violet in methanol. After a proper wash, cells were again incubated in methanol; 100 μL from each well was transferred to a new 96-well plate, and the absorbance was measured at a wavelength of 590 nm, using an ELISA reader (Bio-Tek Instruments, Winooski, VT). Blank values were subtracted from experimental values to eliminate background readings.

Differentiation assay

Cell differentiation assay was performed by alcian blue (Wako Pure Chemical Industries, Osaka, Japan) staining, as previously described.[29] Following crystal violet staining, the cells were washed with methanol and then 3% acetic acid. Cultures were then stained with 1% (v/v) alcian blue in 3% acetic acid, pH 1.0. The cartilage proteoglycans were extracted with 4*M* guanidine hydrochloride (GH), and the bound dye was measured at wavelength of 600 nm, using an ELISA reader (Bio-Tek Instruments). Fresh 4*M* GH served as the blank. Blank values were subtracted from experimental values to eliminate background readings.

Analytical assays

Commercially available assay kits (collagen and glycosaminoglycan [GAG] assay kits, Biocolor, Newtownabbey, Northern Ireland) were used for the measurement of collagen and sulfated GAGs within the cultured cells, as previously described.[30]

Briefly, for the GAG assay, GAG was extracted from the cultured cells using a solvent system of 4*M* guanidine-HCl, 0.5*M* sodium acetate, pH 6, with 1 m*M* benzamidine-HCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 m*M* *N*-ethylmaleimide (NEM). Incubation was carried out at 4°C on an orbital shaker for a 12- to 20-h period. After the extraction, the samples were centrifuged, and blyscan dye reagent (composed of 1,9-dimethyl methylene blue in an organic buffer) was mixed with the supernatant. The GAG–dye complex was collected by centrifugation. The dye bound to the pellet was subsequently solubilized by mixing it with a dissociation reagent. The absorbance of the samples was measured at a wavelength of 656 nm, using a UV spectrophotometer. A calibration solution containing chondroitin-4 sulfate was used to obtain the standard curve for this experiment.

The total collagen concentration (acid- and pepsin-soluble fractions) of the cultured chondrocytes was also measured. The acid-soluble collagen was removed by adding 0.5*M* acetic acid to the cultured cells, followed by centrifugation. The remaining pepsin-soluble collagen was subsequently extracted from the cultured cells. A pepsin solution (1 mg/10 mg tissue sample; Sigma) was added to the cells, and they were incubated overnight at 37°C. Both the acid- and pepsin-soluble collagen samples were further separated for assay by mixing with Sircol dye reagent for 30 min in a mechanical shaker, and the collagen–dye complex was collected by centrifugation. The dye bound to the collagen pellet was solubilized with an alkaline reagent, and the absorbance of the samples was measured at a wavelength of 540 nm, using a UV spectrophotometer. A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for this experiment.

Real-time polymerase chain reaction

To detect the presence of collagen type II and aggrecan, single-stranded cDNA was prepared from 1 μg of total RNA by reverse transcription (RT), using a commercially available First-Strand cDNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Subsequently, real-time polymerase chain reaction (PCR) was done using a LightCycler system with LightCycler FastStart DNA Master SYBR Green I

(Roche Diagnostics, Penzberg, Germany). The LightCycler™- Primer set (Roche Diagnostics) was used for quantitative detection of the collagen type II and aggrecan genes, and also for quantitation of a housekeeping gene, Glyceraldehide-3-phosphate dehydrogenase (GAPDH), according to the manufacturer's instructions. An initial denaturation step at 95°C for 10 min was followed by amplification and extension steps for 35 cycles (95°C for 10 s, 68°C for 10 s, 72°C for 16 s) with final extension step at 58°C for 10 s. The quantification data were analyzed with the LightCycler analysis software (Roche Diagnostics).

Statistical study

Student's t tests were used to assess whether differences observed between the polymers treated and the control samples were statistically significant. For comparison of groups of means, one-way analysis of variance was carried out. When significant differences were found, Tukey's pairwise comparisons were used to investigate the nature of the difference. Statistical significance was accepted at $p < 0.05$. Values were presented as the mean ± SD (standard deviation) except in figure 3. Four samples were run for each case. All experiments were repeated at least twice, and similar results were obtained.

RESULTS

Cell proliferation

Chondrocyte proliferation was quantified by crystal violet staining and expressed as a percentage of the average control value (Fig. 1). Cell proliferation was increased 1.8-fold ($p < 0.05$) in PGA(Sn)-treated cultures compared with that of the control culture, whereas cell proliferation in PGA-treated cultures was almost identical to the DMSO-treated control culture.

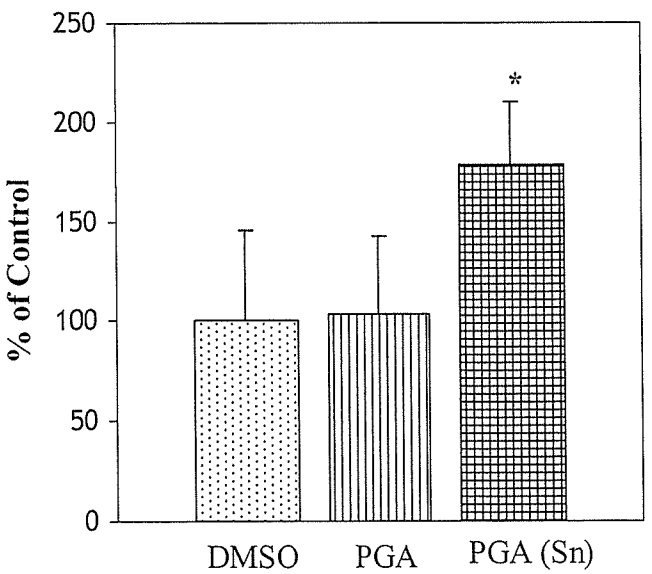

Figure 1. Proliferation of HAC estimated by crystal violet staining. Cell proliferation was significantly increased in PGA(Sn)-cultured chondrocytes compared with that of the control. *$p < 0.05$. All experiments were run in quadruplicate for two separate times.

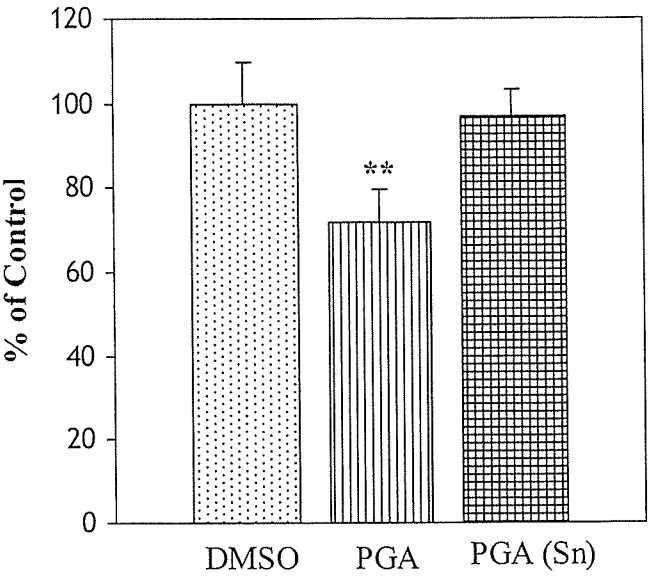

Figure 2. Differentiation of HAC estimated by alcian blue method. Cell differentiation was significantly inhibited in PGA-cultured chondrocytes compared with that of the control. **$p < 0.01$. All experiments were run in quadruplicate for two separate times.

Cell differentiation

Chondrocyte differentiation was estimated by alcian blue staining and the amounts were expressed as a percentage of the average control value, which was calculated as 100%. Chondrocytes treated with PGA revealed a 0.71-fold ($p < 0.01$) decrease in cell differentiation compared with that of the control culture. At the same time, cultures treated with PGA(Sn) showed a slight, but nonsignificant, decrease in cell differentiation (Fig. 2).

Extracellular matrix gene expression

Extracellular matrix gene expression was quantitatively measured by real-time PCR. Here, compared with that of the control culture, the collagen type II gene was more strongly expressed ($p < 0.01$) in PGA(Sn) than in PGA-treated cultured chondrocytes [Fig. 3(A)]. Aggrecan gene expression was inhibited in the latter, but no difference was observed between the former and the control culture [Fig. 3(B)].

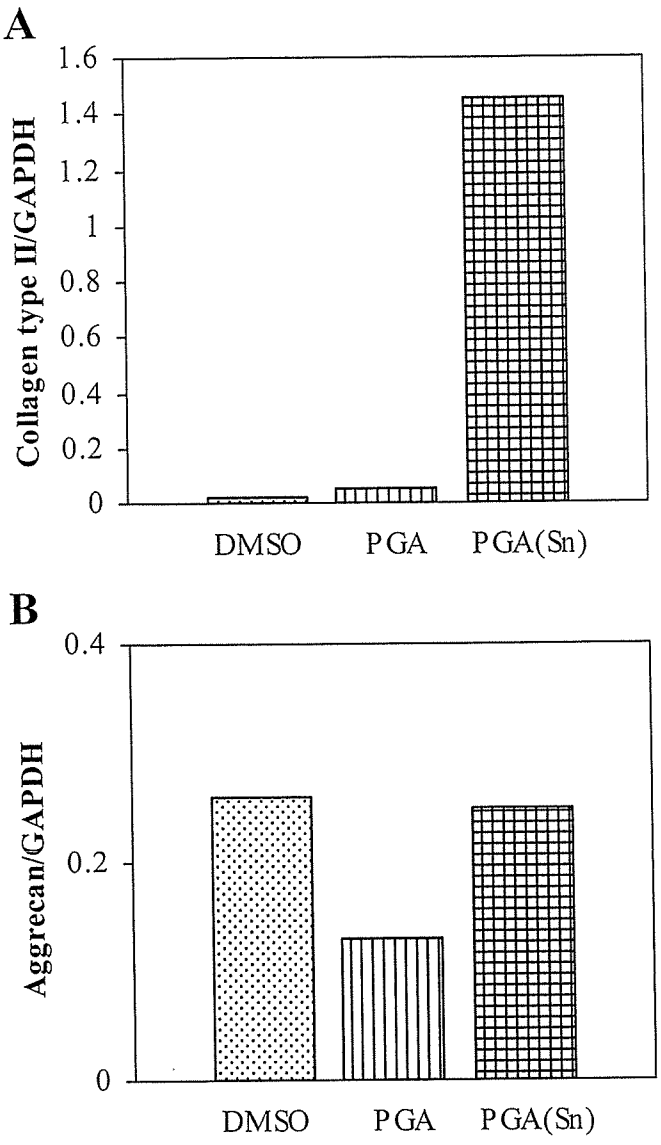

Figure 3. Extracellular matrix gene expression of HAC by real-time PCR. (A) Collagen type II gene was more strongly expressed in PGA(Sn)- than PGA-cultured chondrocytes compared with that of the control culture. (B) Aggrecan gene expression was inhibited in PGA, but no difference was observed between the PGA(Sn) and the control. All experiments were run in quadruplicate for two separate times.

Measurement of collagen type II protein

The amount of pepsin-soluble and cartilage-specific collagen type II protein was increased in both PGA and PGA(Sn) treated chondrocytes on comparing with that of the control culture(Fig. 4). However, this increase was more in the latter than in the former case.

Measurement of total collagen

Quantitative estimations of both acid- and pepsin-soluble total collagen revealed a decrease in PGA(Sn)-treated cultures compared with that of the control

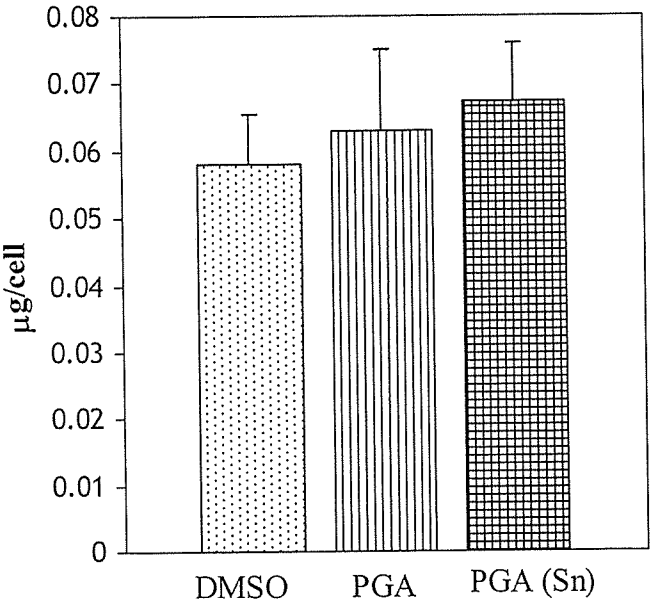

Figure 4. Measurement of collagen type II protein. The amount of collagen type II was increased in PGA(Sn)-treated chondrocytes compared with that of control. All experiments were run in quadruplicate for two separate times.

(Fig. 5). Simultaneously, there was a slight increase in the amount of total collagen in PGA-treated cultures compared with that of the control sample.

Estimation of sulfated glycosaminoglycan concentration

Evaluation of the amount of sulfated GAG showed a decrease in PGA(Sn)-treated cultured cells com-

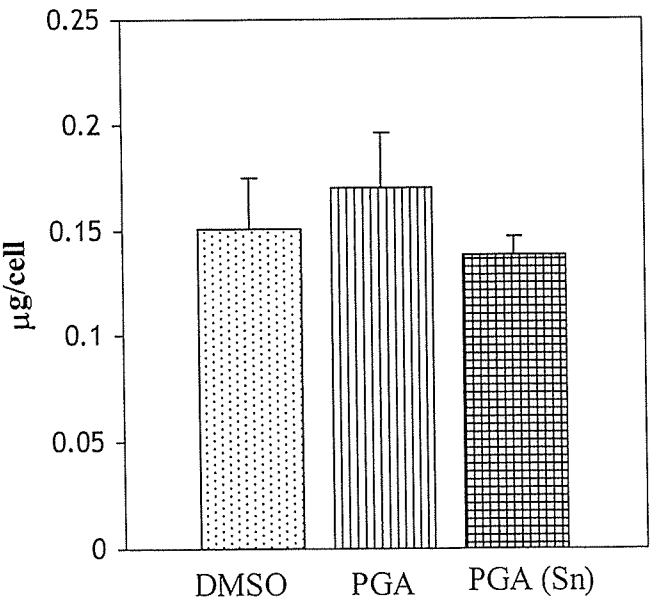

Figure 5. Quantitative estimation of total collagen protein. The amount of total collagen was decreased in PGA(Sn)-treated cultures compared with that of the control. All experiments were run in quadruplicate for two separate times.