特別記事

細胞組織医療機器などの品質・安全性確保について

Quality, Efficacy and Safety of Tissue Engineered Medical Products

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はじめに

2000年12月26日付けの，ヒトまたは動物由来成分を原料として製造される医薬品などの品質および安全性確保についての医薬安全局長通知(医薬発第1314号)において，別添1「細胞組織利用医薬品等の取扱い及び使用に関する基本的考え方」と別添2「ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」の二つの文書が示された。

別添1の「基本的考え方」は，ヒトや動物の細胞・組織から構成される医薬品および医療機器(2005年度から医療用具は，医療機器に名称変更される予定)について，品質および安全性の確保，ならびに細胞・組織の取り扱いに関する科学的および倫理的妥当性を確保するための方策をまとめている。

別添2の指針は，「基本的考え方」に基づき，ヒトの細胞・組織に培養処理などの加工を施して製造される医薬品および医療機器について，品質および安全性の確保のために必要な基本的技術要件を定めており，治験前に厚生労働省に提出する品質および安全性の確認申請に必要な添付資料の内容を示している。

2001年3月28日付けで，「薬事法施行規則の一部を改正する省令等の施行について(細胞組織医薬品及び細胞組織医療用具に関する取扱いについて)」(医薬発第266号)省令および告示が公布され，2001年4月1日より施行されている。

ヒトまたは動物の細胞または組織より構成された医療機器および医薬品に関して，科学技術の進歩に伴う感染症への対策が急務となり，ドナースクリーニング，感染因子の不活化など，ドナーに由来する感染症への対策や，培養などの処理により細胞または組織が有害な性質のものとならないことの確認など，品質および安全性を確保するための特別の対策が必要とされ，改正された。

細胞組織医療機器などの適用範囲

細胞組織医療機器，細胞組織医薬品とは，ヒトまたは動物の細胞または組織より構成される医療機器および医薬品のことであり，動物由来の組織を利用して承認を取得している生体弁や心嚢膜も含んでいる。

改正概要

承認申請書の記載方法の変更(詳細は，最新の薬事法で確認のこと)や，GMP関係省令についても一部改正された。その概要としては，細胞組織医療機器などを製造または輸入するにあたり，細胞もしくは組織由来または製造工程中の感染症などの伝播による危険性を排除し，不適切な製造や取り扱いによる品質および安全性上の問題の発生を防止するために，製造管理と品質管理に必要な要求事項を定めた。

具体的には，

①細胞組織医療機器などの製造所の構造設備の基準への適合，原料の受け入れ，加工処理，製品の保

管などを行う区域について，他区域からの区分，必要な構造および設備の要求。

②加工処理の「加工」とは，疾病の治療や組織の修復または再建を目的として，細胞または組織の人為的増殖，細胞または組織の活性化を目的とした薬剤処理，生物学的特性改変，遺伝子工学的改変，非細胞または非組織成分とのハイブリッド化，カプセル化などを施すことを意味する。

③細胞組織医療機器などについては，生物学的製剤などと同様に，原料として使用するヒト，動物，植物または微生物から得られたものに係る事項や使用動物の規格に関する事項について，製品標準書を作成すること。

④細胞組織医療機器などの製造，保管，出納，ならびに衛生管理に関する記録については，遅発性感染症の危険性を否定し得ないことから，安全性の確保上必要な情報を得るために，少なくとも所定の期間記録を保存することとなっている。すなわち，特定生物由来製品の場合，医療機関での患者使用記録の保管期間は20年間とし，製造業者などでの提供者・製造記録の保管期間は30年間と規定。生物由来製品では，製造業者などでの提供者・製造記録の保管が求められ，ヒト血液成分以外の成分に関する記録は10年間，ヒト血液成分を含む場合のヒト血液成分に関する記録は30年間保管となっている（生物由来製品の特性に応じて保管期間は異なる。最新版の薬務公報に目を通し，現時点での正確な情報を入手すること）。

⑤細胞または組織の取り違えや，細菌，真菌，ウイルスなどの伝播の危険性を避けるために，製造工程において複数のドナーからの細胞・組織を同一室内で同時期に取り扱ったり，交叉汚染を引き起こすような保管方法を取らないこと。さらに，ドナーまたはドナー動物ごとに，細胞や組織，中間製品および製品を管理する必要がある。

⑥原料となる細胞または組織について，表1に基づき，適格なものであることを確認し，その結果に関する記録を作成すること。

⑦「施設」は組織を採取した医療施設もしくは動物の細胞または組織を採取した施設を指す。

⑧「適格性を有する」とは，「細胞組織医薬品及び細胞組織医療用具に関する基準」より以下に述べるいずれの項目にも該当し，原料となる条件を満たしているものを指す。細胞組織医療機器について，薬事法第42条の規定に基づき，品質および安全性確保の観点から，原料または材料となる細胞または組織に関する基準を定めている（2001年3月厚生労働省告示第101号関係）。この基準を満たさない細胞または組織は，品質および安全性についての情報が十分でないことから，製造業者は，これらの細胞または組織を原料または材料として医薬品または医療機器を製造すべきでない。「基準の概要」として，医薬品または医療機器の

表1　原料となる細胞・組織の適格性と記録について

①当該細胞または組織を採取した施設
②当該細胞または組織を採取した年月日
③当該細胞または組織が人に係るものである場合には，ドナースクリーニング（ドナーについて，問診，検査などによる診断を行い，細胞組織医薬品の原料となる細胞または組織を提供する適格性を有するかどうかを判定することをいう）のためのドナーの問診，検査などによる診断の状況。
④当該細胞または組織が動物に係るものである場合には，ドナー動物の受け入れの状況ならびにドナースクリーニング（ドナー動物について，試験検査および飼育管理を行い，細胞組織医薬品の原料となる細胞または組織を提供する適格性を有するかどうかを判定することをいう）のためのドナー動物の試験検査，および飼育管理の状況。
⑤当該細胞または組織を採取する作業の経過（採取する作業経過に関する記録と，採取作業において微生物などに汚染されていない旨が確認できるものであること）。
⑥①から⑤までに掲げるものの他，細胞組織医療機器などの品質の確保に関して必要な事項（製造に使用する試薬に関する試験検査結果を指す）。

原料または材料となる細胞・組織については，必要な衛生管理と人員をもつ施設で採取されていること，ドナースクリーニングが適切に行われていることが確認できること，採取作業が適切に行われていることが確認できること，必要な記録を確認できること，が必要である。ドナースクリーニングの項目など具体的な内容については，個別の製品ごとに異なることから，具体的事項については承認申請書に記録する。

以上が改正概要であるが，紙面の都合上，すべての内容について記載はできない。薬務関連の公報最新版（厚生労働省ホームページ：薬事法；http：//wwwhourei.mhlw.go.jp/~hourei/html/hourei/contents.html，審査管理課関連通知；http：//www.nihs.go.jp/mhlw/tuuchi/index.html）を読み，正確な情報を入手することが重要である。

2003年10月25日，医療機器フォーラム（http：//dmd.nihs.go.jp/iryokiki/）設立記念シンポジウムとして，「Tissue Engineering—開発と評価」に関する講演会を開いた。Tissue Engineeringを含む医療機器の健全な発展を図るために，医療機器の開発，製造および品質管理に係る問題について，産官学の情報交換の場を作ることを目的として設立した。医療機器フォーラムは，毎年1回は定期的に開催（10月予定）する予定である。安全かつ優れた医療機器の開発支援に本フォーラムが有効に活用されることを願っている。

細胞組織医療機器等の製品化のためのガイドライン，環境整備について

医療機器・細胞組織医療機器関連の薬事法の改正が昨年度からスタートしている。その内容は，(1)多様性に富んだ医療機器のリスクに応じた新クラス分類とその承認制度の見直し，(2)細胞組織医療機器が含まれる生物由来製品の感染リスクに応じた安全対策の充実，(3)市販後安全対策の抜本的見直しが急ピッチで進められている。また，第三者認証制度の導入において必要な規格・基準の整備も行われている。本稿では，細胞組織医療機器や新たな制度である生物由来製品の薬事法の改正内容について記載した。

土屋利江

1. 細胞組織医療機器等の薬事法改正について

平成12年12月26日付けでヒトまたは動物由来成分を原料として製造される医薬品等の品質及び安全性確保についての医薬安全局長通知（医薬発第1314号）において，別添1「細胞組織利用医薬品等の取扱い及び使用に関する基本的考え方」と別添2「ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」の2つの文書が示された。

別添1の基本的考え方は，ヒトや動物の細胞・組織から構成される医薬品および医療機器（平成17年度から医療用具は，医療機器に名称変更される予定）について，品質および安全性の確保ならびに細胞・組織の取扱いに関する科学的および倫理的妥当性を確保するための方策をまとめている。

別添2の指針は，「基本的考え方」に基づき，ヒトの細胞・組織に培養処理等の加工を施して製造される医薬品および医療機器について，品質および安全性の確保のために必要な基本的技術要件を定めており，治験前に厚生労働省に提出する品質および安全性の確認申請時に，必要な添付資料の内容を示している。

平成13年3月28日付けで「薬事法施行規則の一部を改正する省令等の施工について（細胞組織医薬品及び細胞組織医療用具に関する取扱いについて）」（医薬発第266号）省令および告示が公布され，平成13年4月1日より施行されている。

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Reform of Biological Products Regulation and Guidelines for Manufacturing Tissue Engineered Medical Products

ヒトまたは動物の細胞または組織より構成された医療機器および医薬品に関して科学技術の進歩に伴う感染症への対策が急務となり，ドナースクリーニング，感染因子の不活化など，ドナーに由来する感染症への対策，培養などの処理により細胞または組織が有害な性質のものとならないことの確認など，品質および安全性を確保するために特別の対策が必要とされ，改正された。

2. 細胞組織医療機器等の適用範囲

ヒトまたは動物の細胞または組織より構成される医療機器および医薬品であり，動物由来の組織を利用して承認を取得している生体弁や心のう膜も含んでいる。

3. 改正概要

承認申請書の記載方法の変更（詳細は，最新の薬事法で確認のこと）や，GMP*関係省令についても一部改正された。その概要は，細胞組織医療機器などを製造または輸入するにあたり，細胞もしくは組織由来または製造工程中の感染症などの伝播による危険性を排除し，不適切な製造や取扱いによる品質および安全性上の問題の発生を防止するために，製造管理と品質管理に必要な要求事項を定めた。

具体的には，

(1) 細胞組織医療機器等の製造所の構造設備の基準への適合，原料の受入れ，加工処理，製品の保管等を行う区域について，他区域からの区分，必要な構造及び設備の要求。

* GMP: 製造許可の要件として製造所の構造設備から製造工程全般にわたる製造管理及び品質管理について製造業者が遵守すべき基準

(2) 加工処理の「加工」とは疾病の治療や組織の修復又は再建を目的として，細胞または組織の人為的増殖，細胞または組織の活性化を目的とした薬剤処理，生物学的特性改変，遺伝子工学的改変，非細胞または非組織成分とのハイブリッド化，カプセル化等を施すこと。

(3) 細胞組織医療機器等は，生物学的製剤等と同様に原料として使用する人，動物，植物または微生物から得られたものに係る事項や使用動物の規格に関する事項について，製品標準書を作成。

(4) 細胞組織医療機器等の製造，保管及び出納ならびに衛生管理に関する記録については，遅発性感染症の危険性を否定し得ないことから，安全性の確保上必要な情報を得るために，少なくとも所定の期間記録を保存することとなっている。即ち，特定生物由来製品の場合，医療機関での患者使用記録の保管期間は20年間とし，製造業者等での提供者・製造記録の保管期間は30年間と規定。生物由来製品では，製造業者等での提供者・製造記録の保管が求められ，人血液成分以外の成分に関する記録は，10年間，人血液成分を含む場合の人血液成分に関する記録は，30年間保管となっている。(生物由来製品の特性に応じて保管期間は異なる。最新版の薬務公報に目を通し，現時点での正確な情報を入手し，確認すること。)

(5) 細胞又は組織の取り違えや細菌，真菌，ウイルス等の伝播の危険性を避けるために，製造工程において複数のドナーからの細胞又は，組織を同一室内で同時期に取扱ったり，交叉汚染を引き起こすような保管方法をとらないこと。更に，ドナー又はドナー動物ごとに細胞や組織，中間製品及び製品を管理する必要がある。

(6) 原料となる細胞又は組織について，後述した内容において，適格なものであることを確認し，その結果に関する記録を作成すること。

(i) 当該細胞又は組織を採取した施設

(ii) 当該細胞又は組織を採取した年月日

(iii) 当該細胞又は組織が人に係るものである場合には，ドナースクリーニング（ドナーについて，問診，検査等による診断を行い，細胞組織医薬品の原料となる細胞又は組織を提供する適格性を有するかどうかを判定することをいう）のためのドナーの問診，検査等による診断の状況。

(iv) 当該細胞又は組織が動物に係るものである場合には，ドナー動物の受入の状況並びにドナースクリーニング（ドナー動物について，試験検査及び飼育管理を行い，細胞組織医薬品の原料となる細胞又は組織を提供する適格性を有するかどうかを判定することをいう）のためのドナー動物の試験検査及び飼育管理の状況

(v) 当該細胞又は組織を採取する作業の経過（採取する作業経過に関する記録と採取作業において微生物等に汚染されていない旨が確認できるものであること）。

(vi) (i)から(v)までに掲げるもののほか，細胞組織医療機器等の品質の確保に関し必要な事項（製造に使用する試薬に関する試験検査結果を指す）。

(7) 「施設」は組織を採取した医療施設もしくは動物の細胞又は組織を採取した施設を指す。

(8) 「適格性を有する」とは，「細胞組織医薬品及び細胞組織医療用具に関する基準」の

以下のいずれにも該当し，原料となる条件を満たしているもの。

細胞組織医療機器について，薬事第42条の規定に基づき，品質及び安全性確保の観点から，原料又は材料となる細胞又は組織に関する基準を定めている（平成13年3月厚生労働省告示第101号関係）。この基準を満たさない細胞又は組織は，品質及び安全性についての情報が十分でないことから，製造業者は，これら細胞又は組織を原料又は材料として医薬品又は医療機器として製造すべきでない。

「基準の概要」

医薬品又は医療機器の原料又は材料となる細胞又は組織については，

必要な衛生管理と人員を持つ施設で採取されていること。
ドナースクリーニングが適切に行われていることが確認できること。
採取作業が適切に行われていることが確認できること。
必要な記録を確認できること。

が必要である。

ドナースクリーニングの項目等具体的な内容については，個別の製品ごとに異なることから，具体的事項については承認申請書に記録する。

4. 生物由来製品に関する制度の概要

(1) 生物由来製品に関する制度の創設について

人又は動物の細胞，組織等に由来する原材料を用いて製造される生物由来製品は，その特性として，原材料の汚染に由来する感染リスク等について，注意を払う必要がある。生物由来というこの共通特性に着目し，原材料採取・製造から市販後に至る，一貫した安全性確保体制を導入し，製品の安全性を図るために創設された。

(2) 生物由来製品に関する制度の主な内容

(a) 生物由来製品及び特定生物由来製品の指定

製品の感染症リスクを考慮した科学的評価に基づき，指定を行い，生物由来製品は約700製品，特定生物由来製品は約280製品について指定し，公表した。

平成15年厚生労働省告示209号「厚生労働大臣が指定する生物由来製品及び特定生物由来製品を定める件」

(b) 生物由来原料基準

生物由来原材料を用いるすべての医薬品等の原材料について，品質・安全性の確保のために，適格性の基準を制定している。

平成15年度厚生労働大臣告示210号「生物由来原料基準を定める件」及び平成15年5月20日医薬発第0520001号「生物由来製品及び特定生物由来製品の指定並びに生物由来原料基準の制定等について」

(c) 血液製剤等の使用記録等の保管期間（前述した。）

(d) 表示

特定生物由来製品，生物由来製品それぞれの容器・包装に識別表示を行う。血液成分を含む特定生物由来製品については，採血国，献血，非献血の別を記載。

(e) 添付文書記載要領

平成15年5月15日付け医薬発第0515005号医薬局長通知「生物由来製品の添付文書に記載すべき事項について」及び平成15年5月20日付け医薬安発第0520004号医薬局安全対策課長通知「生物由来製品の添付文書の記載要領」において，生物由来製品に係る添付文書の具体的な記載要領等を定めている。

(e) 感染症定期報告

平成15年5月15日付け医薬発第0515008号医薬局長通知「生物由来製品に関する感染症定期報告制度について」において，生物由来製品に係る感染症定期報告の具体的な報告方法等について記載している。

(f) 使用対象者への説明並びに記録及び保存

医療関係者にたいし，特定生物由来製品の適正な使用のための必要な事項についての使用対象者への説明を義務づけている。また，特定生物由来製品の遡及調査等を可能とするために，使用の対象者の氏名等の記録及びその保存を義務付けた。

製造業者に対しては，生物由来製品の遡及調査等を可能とするため，販売等を行った生物由来製品に関する記録及びその保存を義務付けている。

(g) 製造業者等の生物由来製品製造管理者の設置要件の規定

生物由来製品の製造業者等は，生物製品製造管理者を設置しなければならない。その承認の対象者は，以下のように規定されている。

(i) 医師，医学の学位を持つ者

(ii) 歯科医師であって細菌学を専攻した者

(iii) 細菌学を専攻し，修士課程を修めた者

(iv) 大学等で微生物学の講義及び実習を受講し，修得した後，3年以上の生物由来製品もしくはそれと同等の保険衛生上の注意を要する医薬品，医療用具等の製造等（治療薬として製造する場合も含む）に関する経験を有する者

5. 医療機関からの副作用等報告制度：医師・薬剤師等の医薬関係者から直接厚生労働省に報告される副作用・不具合又は感染症報告の報告事項を規定

平成15年5月15日付け医薬発第0515014号医薬局長通知「医療機関等からの医薬品または医療用具についての副作用，感染症及び不具合報告の法制化に伴う実施要領の制定について」により，製造業者のみならず，医療機関からの報告事項について規定された。

6. おわりに

紙面の都合上，すべての改正内容について記載はできない。薬務関連の公報最新版

厚生労働省ホームページ：薬事法
http://wwwhourei.mhlw.go.jp/~hourei/html/hourei/contents.html
審査管理課関連通知 http://www.nihs.go.jp/mhlw/tuuchi/index.html

を読み，正確な情報を入手することが重要である。

2003年10月25日医療機器フォーラム（http://dmd.nihs.go.jp/iryokiki/）設立記念シンポジウムを開催し，「Tissue Engineering—開発と評価」に関する講演会を開いた。全国から多くの方々が参加された。医療機器フォーラムは，Tissue Engineering（平成17年度から細胞組織医療機器と名称される）を含む医療機器の健全な発展を図るために，医療機器の開発，製造および品質管理にかかわる問題について，産官学の情報交換の場をつくることを目的として設立した。医療機器フォーラムは，毎年1回は定期的に開催（10月予定）する予定である。安全かつ優れた医療機器の開発支援に本フォーラムが有効に活用されることを願っている（医療機器フォーラムのホームページ：http://dmd.nihs.go.jp/iryokiki/）。

高分子学会は，生物由来の感染因子による汚染の可能性が少ない材料を合成できる研究者で構成されている。生分解性高分子を主たる細胞の足場とする細胞組織医療機器を発展させるためには，有効性，安全性の高い材料を開発していただくことが重要なポイントとなる。製品の上市化に有望な新規材料を開発された場合には，是非，医療機器フォーラムに参加・発表していただき，有効で安全な医療機器の開発を促進できれば，と願っている。

25

EVALUATION OF THE IMMUNO-PROTECTIVE EFFECTS OF THE NEW-TYPE OF BAGS USING ELISA- AND FACS-ANALYSIS.

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Abstract

This study investigated the usefulness of modified polyurethane (MPU) coating micropore membrane bags for diminishing the immunological responses following organ or tissue transplantation in allogeneic setting. Spleen from Brown Norway (BN) rats (donor) were placed into the peritoneal cavity of Lewis rat (recipient) either directly or inside of MPU coated bags. Lewis rat with Sham's operation served as control. After 12 and 24 weeks, cytokines of IL –4, IL –13, TNF –α and IFN -γ, and flow cytometric evaluations for $CD4^+$ and $CD8^+$ cells of the recipient blood were carried out. TNF –α levels proved polyurethane coating effective in reducing inflammatory reaction at 12 weeks. Twelve week IFN -γ and, $CD4^+$ and $CD8^+$ cells indicated that graft-versus-host-reaction (GVHR) took place but polyurethane coated bag did not prevent or reduced this reaction. Thus, this study shows that MPU coating might be functional in preventing inflammatory reaction but is not useful for preventing GVHR.

1. Introduction

Polyurethanes form a versatile and useful class of polymers. As biomaterials, their uses have included the artificial heart, catheters, and synthetic blood conduits.[1] Rejection and graft-versus-host disease are common as the solid organ transplantation and potential immunosuppressive drugs are routinely used after organ transplantation. Unfortunately, the risks of opportunistic infections, lymphoblastic malignancy and metabolic complications are frequently associated with immunosuppressive therapy.[2-4] However, following most of the allotransplants, cell traffic seems to be a striking event with all transplants.[5] Donor cells leaving the solid organ and recipient cell entering it include passenger leucocytes that were shown to be the main cause of allograft immunogenicity. TNF -α and IFN -γ are pro-inflammatory Th1-type cytokines, mediate cellular immune responses and have been shown to be involved in allograft rejection. On the other hand, Th2-cytokine IL –4 and IL -13 played their role in promoting graft survival has been suggested in animal models.[11] Donor $CD4^+$ and $CD8^+$ cells that traffic to recipient, play an important role in the immunogeneic outcome of the host tissue. The objective of this examination was to evaluate the effect of modified polyurethane (MPU) coated micropore membrane bag to eliminate or reduce the immunogenicity in an *in vivo* study using rat model.

2. Materials and Methods

2.1. Polyurethane coated bag

Bags with a size of 2 x 1.5 cm were made from MPU coated micropore membrane. The outer surface of the bag were coated by MPU. Bags were sterilized and turned into the hydrophilic characteristic bags by gradually dipping in 100 %, 90 %, 80 %, 70 %, 50 % and 25 % ethanol aquous solution. Each step lasted for a half day and finally washed by distilled water and phosphate buffer saline.

2.2. Animals and Experimental Groups

K. Yagasaki et al. (eds.), Animal Cell Technology: Basic & Applied Aspects, Volume13, 277-280.

Eight-week-old Lewis and Brown-Norway (BN) female rats were obtained from Charles River Japan Inc. Kanagawa, Japan. Rats were maintained in an air-conditioned animal facility at the national Institute of Health Sciences. The principles of laboratory animal care (according to NIH Publication No. 85-23, revised 1985) were carefully followed in this study. The rats were fed with a commercial diet and water *ad libitum*, pre- and post-implantation periods. Spleens from BN rats (donor) were placed into the peritoneal cavity of Lewis rats (recipient) either directly or inside of MPU coated bags. Lewis rat with Sham's operation served as control. In another group, only MPU coated bag was placed into the peritoneal cavity.

2.3. Operative Procedures

The donor, under sedation with ether was anesthetized by intraperitoneal administration of pentobarbital sodium (20 mg/ kg body weight). After proper sterilization, peritoneal cavity was opened through a ventral midline incision. From the BN rats spleens were removed and washed with PBS and immediately placed into the peritoneal cavity of Lewis rats, either directly or inside of a MPU coated bag containing 1 ml of RPMI 1640 medium (Dutch modification, Gibco BRL, Life Technologies Ltd., Paisley, Scotland). In bag group, only bag containing 1 ml of the medium was placed into the Lewis peritoneal cavity and in control group, Sham's operation was performed. After the designated experimental period, rats were anesthetized in the same fashion as the initial operation. On opening the abdomen, spleens were collected (12 weeks post implantation only) and blood was collected from the descending abdominal aorta of recipient and then the rats were sacrificed.

2.4. Cytokines Assay

After 12 and 24 weeks of implantatiohn, blood collected from the recipient was centrifuged and supernatant were stored at –80°C until cytokines were measured. Cytokines levels of IL- 4, IL – 13, TNF- α and IFN- γ were measured using conventional ELISA assay (Biosource International, Inc., CA, USA) according to the manufacturer's instruction. Cytokine concentrations were calculated using manufacturer supplied cytokine standards and expressed in pg/ml.

2.5. Flow Cytometry

The analysis for $CD4^+$ and $CD8^+$ cells was carried out by flow cytometry. Anti-coagulant treated venous blood samples were analyzed with two-color flow cytometry to determine the percentages of $CD4^+$ and $CD8^+$ T cells. Briefly, 100 µl of heparin treated venous blood were incubated with 20 µl of the indicated FITC-labeled anti-CD4 and PE-labeled anti-CD8 mAb, vortexed vigorously and incubated at room temperature for 45 min. Following washing with 4 ml PBS two times, and futher washing with 1 ml of immuno-lyse working solution and 250 µl of fixative was added within 30 sec to 2 min. After washing by PBS solution two times, the pelleted cells were analyzed with an EPICS XL II cytometer (Beckman-Coulter, Margency, France). The analysis was focused on lymphocytes, identified by their forward and right angle scatter features. At least 10000 events were collected in the lymphocyte gate and analyzed.

2.6. Antibodies

Rat mAb FITC –conjugated anti CD4 (IM3056) and PE –conjugated anti CD8 were used in this study. These mAb and their isotype-matched negative control mAb [mouse IgG1 –FITC (IM0639) and IgG1 –PE (IM0670)] were purchased from Beckman Coulter Immunotech (Marseille, France).

2.7. Statistical analysis

Data are presented as mean ± SD. Values of different experimental groups were analyzed with a paired t test. Differences were considered statistically significant at $p < 0.05$).

3. Results

3.1. Macroscopic findings

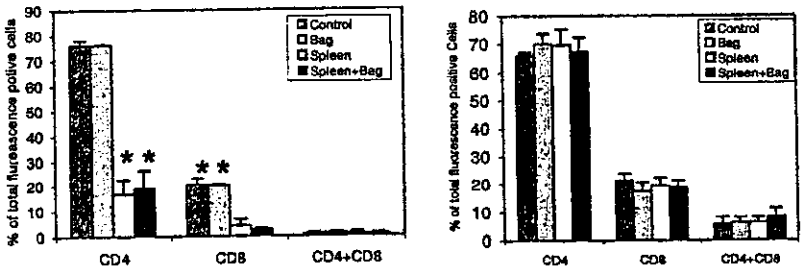
At 12 weeks, spleens implanted either directly into the peritoneal cavity or inside of MPU coated bags were collected and observed. The spleens shrinked and weight of the spleens that were directly implanted into the peritoneal cavity was more reduced as compared with the weight of the spleens that were implanted inside the polyurethane coated bags.

3.2. Cytokines Expression

	12 week				24 week			
	C	B	S	BS	C	B	S	BS
TNF-α	-	↑	↑↑	↑	-	-	±	±
IL-4	-	-	-	-	-	-	-	-
IL-13	-	-	-	-	ND			
IFN-γ	-	↓	↓	↓	ND			

Table 1. C, control; B, bag; S, spleen; BS, spleen inside bag. N.D., not done; ↑, increase; ↓, decrease; -, no increase than control; ±, no or almost no increase than control..

Expressions of various cytokines are summarized in Table 1. Expressions of TNF -α at 12 post-implantation weeks were increased in all groups as compared to control group. Highest expression of TNF -α was detected in spleen group, followed by the group of spleen inside of the bag. There was no or almost no increase in TNF -α expression among various groups as compared to control at 24 post-implantation weeks. IL -4 expression at 12 and 24 post-implantation weeks and IL -13 expression at 12 post-implantation weeks were similar among all experimental groups. Expression IFN -γ at 12 post-implantation weeks were decreased in all experimental groups when compared with that in the control group.



(a). 12 weeks implantation (b). 24 weeks implantation

Figure 1. FACS analysis for $CD4^+$ and $CD8^+$ lymphocytes of recipient blood. (a) 12 weeks implantation, (b) 24 weeks implantation. Significantly different from the controls at $^*p < 0.05$.

3.3. Flow Cytometry

At 12 post-implantation weeks, number of $CD4^+$ cells were significantly decreased in spleen-group and the group of the spleen involved inside of the bag (Spleen+Bag) and the maximum decrease was found in spleen group. $CD8^+$, and both $CD4^+$ and $CD8^+$ cells were also decreased in spleen gruoup and the group of spleen involved inside of the bag [Fig. 1(a)]. There was no difference in $CD4^+$, $CD8^+$, and both $CD4^+$ and $CD8^+$ cell numbers among various groups at 24 post-implantation weeks [Fig. 1(b)].

4. Discussion

In allorecognition, the specific activation of T cells is initiated by the binding of foreign alloantigen and/or host MHC molecule complex to T-cell receptors. The activated helper T cells secrete lymphokines, which induce the proliferation and maturation of activated cytotoxic T cells. In addition, helper T cells secrete IFN -γ, which induces cell surface expression of MHC antigens and activates macrophages. At 12 weeks following spleen implantation, inflammation was noted in all groups except control group and the polyurethane bags exhibited some protective effect for the implanted organ. The donor spleen T cells were cytotoxic to recipient T cells and resulted a decrease in secretion of IFN -γ in 12 week post-implantation recipient serum and decreased the number of recipient $CD4^+$ and $CD8^+$ T cells. At 24 weeks of post transplantation the spleens placed directly into the peritoneal cavity were absorbed by phagocytosis and those inside the bags were completely necrosed. Therefore, there was no effective source of donor cytotoxic T cells to act on recipient T cells and no difference of $CD4^+$ and $CD8^+$ T cells number was observed among different groups at 24 weeks of post implantation. No difference in cytokines TNF -α and IL –4 among different groups of implantation were consistent with the fact that there was no effect of donor T-cells. Also, no inflammatory reaction was observed at 24 weeks of post-implantation. As to the best of our knowledge this study is the first to investigate the role of MPU coating in preventing inflammatory/immunological responses in allogeneic setting, we could not compare our data with other study. However, our data are in agreement of our in vitro study where polyurethane coated bag increased the viability of donor bone-marrow lymphocytes and diminished immunological responses by recipient blood lymphotytes (unpublished data).

This study suggests that MPU coated bag is helpful in diminishing the inflammatory response but played no part in preventing the immunological-reaction in allogeneic settings. Further, this analysis serves as an early indicator in diminishing inflammatory reactions and more experiments are necessary to improve our knowledge of the potential role of MPU coating as anti-inflammatory therapeutic agent or as a coating agents of biomaterials.

5.Acknowledgements

We are grateful to the support of Health and Labour Sciences Research Grants, Research on Advanced Medical Technology, Ministry of Health, Labour and Welfare and Japan Health Sciences Foundation.

6. References

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DIFFERENT EXPRESSION OF GAP JUNCTIONAL PROTEIN CONNEXIN43 IN TWO STRAINS OF MICE AFTER ONE-MONTH IMPLANTATION OF POLY-L-LACTIC ACID

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Abstract. The implantation of a biomaterial often induces host inflammatory responses. Some adverse effects by the biomaterials, such as poly-L-lactic acid (PLLA) and polyurethanes (PUs) were reported in animal experiments. PLLA produced tumorigenicity in rats after long-term implantation. The purpose of this study was to determine the in vitro effect of PLAO3 (high-molecular weights of PLLA) and PU8 (PTMO/MDI/BD) on the function of the normal human dermal fibroblasts (NHDF) and the in vivo effect of PLAO3 on the function of the cells originated from the subcutaneous tissue in the two female mouse strains, BALB/cJ and SJL/J. The results with Scrape-loading and dye transfer (SLDT) assay, Western Blot and RT-PCR analysis clearly demonstrated that gap-junctional intercellular communication (GJIC) and the expression of Cx43 were significantly suppressed in PLAO3-implanted group of BALB/cJ mice in compared to the control mice. While, no significant difference was found in GJIC and the expression of mRNA level but a little bit difference was observed in the Cx43 protein expression between the SJL/J implanted and the control mice. We considered that the PLAO3 suppressed irreversibly gap junctional protein connexin43 at the earlier stage after implantation and the suppression of connexin43 gene-expression might play a vital role in the inhibition of GJIC and thus promotes the tumorigenesis.

Keywords: Poly-L-lactic acid, GJIC, Connexin43.

K. Yagasaki et al. (eds.), Animal Cell Technology: Basic & Applied Aspects, Volume13, 481-485.

1. INTRODUCTION

Some adverse effects caused by the biomaterials, such as poly-L-lactic acid (PLLA) and polyurethanes (PUs) were reported in animal experiments [1]. PLLA produced tumorigenicity in rats after long-term implantation. PUs were also used for implant applications because of their useful elastomeric properties and high tensile strength, lubricity, and good abrasion resistance. However, different kinds of PUs induced various tumor incidences in rats [2]. All tumors have been generally viewed as the outcome of disruption of the homeostatic regulation of cellular ability to response to extra-cellular signals, which trigger intra-cellular signal transduction abnormally [3]. We have hypothesized that the different tumorigenic potentials of PLLA and PUs are caused mainly by the different tumor-promoting activities of these biomaterials. In the present study, we investigated the effect of PLAO3 (a high-molecular weight PLLA) and PU8 on the normal human dermal fibroblast (NHDF). Our present results showed that the PLAO3 inhibited GJIC, whereas PU8 did not inhibit GJIC after 17 days culture on these materials. These findings inspired us to investigate the role of PLAO3 on the subcutaneous tissue of the two different responder strains of BALB/cJ and SJL/J mice.

2. MATERIALS AND METHODS

2.1. ***NHDF Cell culture:*** The NHDF cells were obtained from Asahi Techno Glass (Tokyo, Japan), and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5 % CO_2 atmosphere at 37°C.

2.2. ***Animals:*** Five-week-old female BALB/cJ and SJL/J mice were obtained from Charles River (Japan).

2.3. ***Implantation of PLAO3:*** PLAO3 (20 X 10 X 1 mm) was obtained from Shimadzu Co. Ltd., and sterilized by ethylene oxide gas prior to use. Sodium pentobarbital (4 mg/kg) was intraperitoneally administered to the mice. A dorsal incision of approximately 2 cm was made, opposite sites from the incision a subcutaneous pocket was formed by blunt dissection, and one piece of PLAO3 was placed in the pocket. The incision was closed with silk thread. In both strains, Sham's operation group served as controls. After 30 days, mice were sacrificed and subcutaneous tissues were obtained for subsequent culture.

2.4. ***Cell culture of subcutaneous tissues:*** The subcutaneous tissues were maintained in

minimum essential medium (MEM) supplemented with 10% FBS in a 5 % CO_2 atmosphere at 37°C. Cells were collected by trypsinization after adequate growth.

2.5. ***Giemsa staining:*** When cells reached confluence in tissue culture dishes, cells were fixed and stained with giemsa solution. Cells morphology was determined under an inverted light microscope.

2.6. ***Scrape-loading and dye transfer (SLDT) assay for detection of GJIC:*** Confluent monolayer cells, after rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline [PBS (+)] were loaded with 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR, USA)/PBS (+) solution and scraped immediately with a sharp blade. After incubation for 5 min at 37°C, cells were washed three times with PBS (+) and the extent of dye migration length was measured using fluorescence microscope.

2.7. ***Western Blot analysis:*** Cells were lysed directly in 100 µl of lysis buffer (50 mM Tris-Hcl, pH 6.8, 2% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride). Equivalent protein samples were then prepared in 7.5 % SDS-PAGE sample buffer containing 2-ME and loaded on 7% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Cx43 protein was detected by anti-Cx43 polyclonal antibodies and ECL system.

2.8. ***RT-PCR analysis:*** Total cellular RNA was isolated from cultured cells in Trizol reagent (Life Technologies, Inc.) following the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA by reverse transcript (RT) using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech). Amplification was performed in a volume of 25 µl containing 1 µl of cDNA, 10 pmol of each primer, 0.625 unit of *Taq* polymerase (Promega, Madison, WI, USA) and 0.2 mM of each deoxynucleotide triphosphate. The amplified product was electrophoresis using 1.5% agarose gel and visualized with SYBR Green. GAPDH gene was amplified as internal control.

3. RESULTS

NHDF cells: Giemsa staining showed that the NHDF cell cultures predominantly formed a uniform monolayer of cells. All cultures maintained the elongated shape of NHDF cells. There was no difference in morphology among the control, PLAO3 and PU8. In SLDT, the GJIC was significantly inhibited in PLAO3-exposed NHDF cells in

compared to the controls. On the contrary, no difference was observed between the PU8 implanted and the control groups.

Mouse cells: Inverted light microscopy and Giemsa staining showed that the mouse cells in cultures formed a cis-cross pattern and caused decreased contact inhibition in BALB/cJ control group (Figure 1A). On the other hand, in SJL/J control group, cells were parallel and maintained the contact inhibition (Figure 1C). All cells in the implanted groups of both the strains, showed cis-cross pattern and the cells were piled up in BALB/cJ group more than in SJL/J group (Figure 1B and 1D).

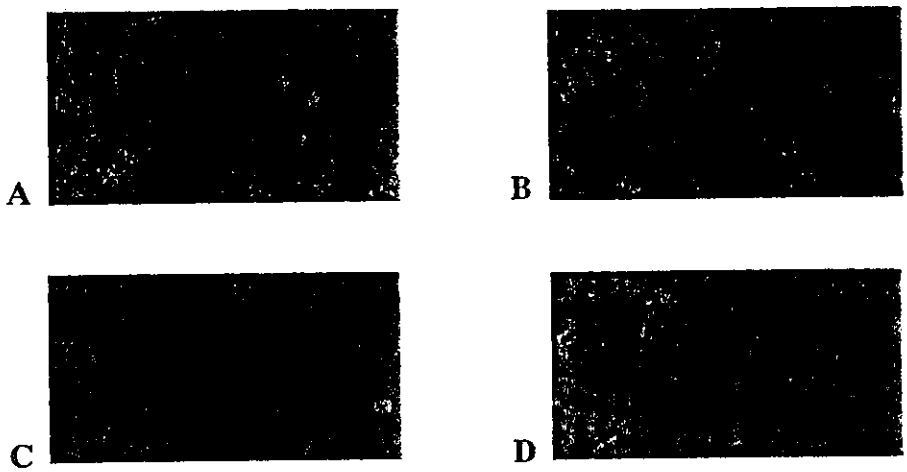

Figure 1 **Mice cells morphology. A: BAJB/cJ control, B: BALB/cJ implanted, C: SJL/J control and D: SJL/J implanted.**

In SLDT, the GJIC was significantly inhibited in PLAO3-implanted BALB/cJ cells in compared to BALB/cJ controls. No difference was observed between the PLAO3-implanted SJL/J and its controls. To clarify the cause, we also examined the mRNA and protein expression levels of connexin43 gene and found that the mRNA and protein expression were suppressed in PLAO3-implanted BALA/cJ mice in compared to BALA/cJ controls. No difference was observed between the PLAO3-implanted SJL/J and SJL/J controls.

4. DISCUSSION

Many factors, that caused tumorigenesis were known, we especially paid attention to the inhibition of the GJIC in the PLAO3-exposed cells. PLAO3 is a widely used

biomaterial for medical and surgical implants. Gap junctions are transmembrane channels that allow the cell-cell transfer of small molecules and are composed of protein subunits known as connexin; at least 19 connexins exist and they are expressed in various kinds of tissues of rodents. Several tumor promoters have been shown to inhibit GJIC by phosphorylation modification of connexin proteins. Connexins are essential proteins to maintain the gap junctional channel [4]. To understand the mechanisms of tumorigenesis induced by PLAO3, we paid attention to the inhibitory effects on GJIC. GJIC is important for normal differentiation of the cells such as neurons and osteoblasts. In the present study, the GJIC was inhibited in PLAO3-exposed NHDF and -implanted BALB/cJ mouse cells. This perturbed gap junction is most likely to play the major role in the PLAO3-induced tumorigenesis. Our results also showed that the mRNA and protein expression of connexin43 gene were suppressed in PLAO3-implanted BALB/cJ mice. Together with these results, we speculated that the inhibitory effect of PLAO3 on GJIC might be due to the alteration in the connexin43 protein. The post-translational modification and decrease in the connexin43 protein has been shown to be involved with impaired GJIC and could be associated in tumorigenesis mechanism. All experiments will be further analyzed at 6 and 12 months after PLAO3 implantation and these experimental data will give us the basic information that are useful for understanding the adverse event induced by medical and surgical implants.

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BIOCOMPATIBLE BIOMATERIALS FOR THE HUMAN CHONDROCYTE DIFFERENTIATION ESTIMATED BY RT-PCR METHOD.

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Abstract

Biocompatibility of the biomaterials for the differentiation of the human articular chondrocytes were estimated by reverse transcription-polymerase chain reaction (RT-PCR). We used five biodegradable polymers for culturing with human articular chondrocytes. In addition to these five materials, we also estimated aquous type of fullerene, namely C60 dimalonic acid (C60DMA). Cultures were carried out using micromass culture method for 4 weeks. Collagen type II, aggrecan and connexin43 gene levels were estimated using RT-PCR methods. Among the biomaterials, Poly glycolic acid (PGA) showed the highest expression level of the collagen type II gene. On the contraty, C60DMA showed the lowest expression level among six kinds of test substances. In the case of the aggrecan gene, PGA also showed the highest levels, and C60DMA showed the lowest ones. However, the expression patterns of the connexin 43 gene were different from previous two genes. Using the multi regression analysis was carried out between differentiation and these three gene expression levels. There was a high correlation between cellular differentiation and three gene expression levels.

1. Introduction

Properties of degradation of scaffolds are the important character in the long-term success of a tissue-engineered cartilage construct. The biodegradable polymers hold the additional advantage that the cartilage tissue, with the biodegradation of the polymers, may gradually replace the space occupied by the scaffolds. Extensive studies have been carried out using bioreabsorbable materials. However, most of those studies used animal cells, whereas little information is available on the chondrogenic effects of these materials with human articular chondrocytes (HAC). The biocompatibility of the biodegradable polymers using human articular cartilage in a micromass culture system was studied. In the present in vitro micromass study, we investigated the biocompatibility of a synthetic biodegradable materials and a fullerene derivative of C60 dimalonic acid (C60DMA) as the indication of the cellular proliferation, differentiation and the expression level of 3 genes such as collagen type II, aggrecan and connexin43, estimated by RT-PCR method.

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2. Materials and Methods

Cell and Materials

Chondrocyte growth medium and HAC were commercially obtained from BioWhittaker, Inc. (Walkersville, MD, USA). Chondrocytes growth medium contains bovine insulin, basic fibroblast growth factor, insulin like growth factor-1, transferring, gentamicin sulfate and fetal bovine serum (5% v/v). PGA (Mw = 3,000) and PLGA (Mw = 5,000) were purchased from Nakalai Tesque Inc. (Kyoto, Japan) and, PGCL (Mw = 3,000) was from Taki Chemical Co (, Japan). P(LA-CL)25 Mw = (10,000) , PCL (Ti) (Mw = 130,000) and fullerene C60-dimalonic acid (C60 DMA) were synthesized in our laboratoty.

Cell culture

In vitro high-density micromass cultures of HAC were initiated by spotting $4x10^5$ cells in 20 µl of medium onto each well of 12-well microplates for tissue culture (Costar ® Type 3513, Corning Co. Ltd., NY, USA) and PCL(Ti) coated glass wells (diameter, 22mm). After two hours of cell spotting in a 5 % CO_2 incubator at 37°C, the wells were flooded with chondrocyte culture media (2 ml/well). Media were supplemented with DMSO (0.8 µl/ml), PGA (50 µg/ml), PGCL (50 µg/ml), PLGA (50 µg/ml), P(LA-CL)25 (50 µg/ml), and fullerene C60 DMA (60 µg/ml), respectively. HAC cultured on tissue culture polystyrene but not exposed to any biomaterials served as a control. The media were changed in every 3 days and the cultures were continued for 4 weeks.

Proliferation assay

Cell proliferation was quantitatively measured by alamar blue (Biosource, International, Inc, Camarillo, CA) assay after 4 weeks of culture as previously described.[1]

Differentiation assay

Proteoglycans are typical contents of the cartilage matrix. The extent of chondrogenesis was determined by staining the cartilage specific proteoglycans with alcian blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as previously described.[2]

RNA harvest

After the designated 4 weeks culture period, RNA was extracted from all matrices except PCL(Ti) matrix. For PCL(Ti) matrix, we did not have enough samples for RNA harvest as cells from 50 % of the cultured wells were detached over night following cell spotting. Total celluar RNA was extracted from cultured cells of four wells (for each material) in 0.5 ml Trizol reagent (Life Technologies, Inc., Frederick, MD, USA) according to manufacturer's instruction.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The matrix molecules probed as part of this study was collagen type II and aggrecan. The gap junction protein gene of Cx43 was also studied. The single strand cDNA was prepared from 1 µg of total RNA by reverse transcription (RT) using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After proper optimization of PCR condition, subsequent PCR was performed with 4 µg of cDNA in a 20 µl reaction mixture (10 x PCR buffer 2 µl,

dNTP 1.6 μl, forward and reverse, each primer 0.4 μl, Taq DNA polymerase 0.1 μl and rest of the amount of distilled water). The codon sequence used for the primer sets was as follows:

Collagen type II: forward 5'-GGCAATAGCAGGTTCACGTACA-3'
reverse 5'-CGATAACAGTCTTGCCCCACTT-3'

Aggrecan: forward 5'-TCGAGGACAGCGAGGCC-3'
reverse 5'-TCGAGGGTGTAGCGTGTAGAGA-3'.

Connexin 43 (Homo Sapiens):
forward 5'-ATGGGTGACTGGAGCGCCTTAGGCAAACTC-3'
reverse 5'- GACCTCGGCCTGATGACCTGGAGATCTAG -3'

The polymerization of GAPDH was accomplished by 25 cycles with the corresponding PCR program. Electrophoresis of PCR products was done on 3% agarose gel for the visualization of collagen type II and aggrecan and, on 1% agarose gel for Cx43 after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). The relative intensity of signals from each lane was analyzed with a computerized scanner. For relative quantitation, the signal intensity of each lane was standardized to that of a housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH):

forward 5'-CCCATCACCATCTTCCAGGAGCGAGA-3'
reverse 5'-TGGCCAAGGTCATCCATGACAACTTTGG-3'.

3. Results

Cell proliferation assay

The cell proliferations of PGA, PGCL and PLGA were fairly parallel as that of control cell proliferation. The cell proliferation of P(LA-CL)25, PCL(Ti) and fullerene C-60 DMA were significantly inhibited as compared to control. The values of cell proliferation for the samples exposed to PGA, PGCL, PLGA, P(LA-CL)25, PCL(Ti) and fullerene C-60 were 101, 102, 104, 93, 84, and 93 %, respectively.

Proteoglycan synthesis

Intensity of alcian blue staining was found to be higher in PGA, PGCL and PLGA containing cultures than that was found with the control culture. Among the biomaterials, PGA caused a significant 3.1 fold increase of cell differentiation when compared to control ($p < 0.05$).

Extracellular matrix genes expression

RT-PCR analysis showed that all matrices consistently expressed collagen type II gene and PGA matrix had the strongest induction. Slight increase expressions of collagen type II gene were noted with PGCL and PLGA matrices. Expression of collagen type II gene in P(LA-CL)25 was faint and in fullerene C60 DMA was almost nil. PGA matrix showed the strongest induction of aggrecan gene. Aggrecan gene expressions were decreased in PLGA and P(LACL)25 matrices.

Expression of gap junction protein connexin 43 gene

PGA induced the highest level of Cx43 mRNA expression and moderate level of expression was noticed in PLGA treated culture. A faint expression in P(LA-CL)25 and almost nil expression in flurrene C60 DMA treated cultures were observed.

Multi-regression analysis

Using the multi regression analysis, correlation was investigated between the differentiation estimated by alcian blue method and three genes expression levels. There was a high correlation between the cellular differentiation and three gene expression (correlation coefficient is 0.96) (Fig.1). Especially, two kinds of expression levels of aggrecan, and connexin 43 genes, were found to be critical factors for estimating the extent of cellular differentiation of human articular chondrocytes(Fig. 1).

4. Discussion

During differentiation, chondrocytes secrete extracellular matrix (ECM) molecules characteristic of cartilage, such as type II collagen, aggrecan, and link protein, offering an environment that preserves the chondrocyte phenotype. Therefore, chondrocyte are defined both by their morphology and ability to produce these characteristic ECM. Collagen type II is regarded as the most important component among the ECM molecules. Previous study detected type II collagen as early as 7 days after beginning 3-D culture and at 21 days, the matrix of the entire aggregate contained type II collagen.[3] Among the ECM molecules, aggrecan is a major proteoglycan[4] and had been reported that in chick cartilage, aggrecan starts to be expressed at embryonic day 5 in limb rudiments, continues through the entire period of chondrocyte development, and remains a biochemical marker of the cartilage phenotype thereafter.[5] In this study, we have well demonstrated cell differentiation with the formation of cartilaginous nodules on culture plate, by alcian blue staining, which is commonly used for identification of cartilage, and by expression of ECM molecules collagen type II and aggrecan. The morphology after the designated culture period revealed that cells aggregated on the culture plate and resulted in the formation of cartilaginous nodules. The greatest cell differentiation, 3.1-fold increase of the controls was found in the sample treated with PGA. The potencies of cell differentiation after 4 weeks of culture from most to least were in the following order; PGA>> PLGA > PGCL > Cont. = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C60 DMA. The increased cell differentiation with PGA and PLGA matrices are in agreement with our previous findings in micromass culture system[1], however, in this study we have included the matrix genes expression of these materials. Results of the present study confirmed PGA and PLGA as useful scaffolding matrices for cartilage tissue engineering, and knowledge with other matrices will further contribute to develop improved cartilaginous constructs for future clinical implants. In this study, RT-PCR analysis showed that the mRNA level of x43 gene expression was consistent with the chondrogenic differentiation in the presence of different biomaterials. Our findings of Cx43 expression by chondrocytes are in agreement of previous study that reported expression of functional gap junctions by chondrocytes isolated from adult articular cartilage[6]. Gap junction mediated intercellular communication is critically involved in the development of cartilage during differentiation[7].

In this study, the data of cell differentiation by alcian blue and, observed expression of collagen type II, aggrecan and Cx43 suggest that the process of cell differentiation might be due to the interconnection of cells by means of gap junction along with other molecular mechanism. However, the specific association of gap junction in the process of chondrogenic differentiation and the cell signaling processes remains unexplored. Future studies are required to analyze the specific role that the gap junction proteins have in chondrocyte differentiation.

5. Acknowledgement

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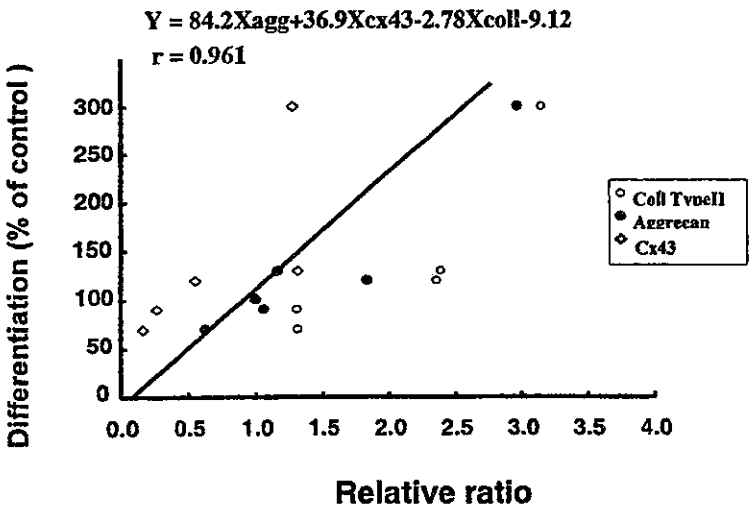

Fig.1.Relationship between the differentiation and the expression levels of three genes of collagen typeII, aggrecan and connexin43 using multi-regression analysis.