ity of HeLa S3 cells, and is faster than traditional animal models using nude or SCID mice.

In cell-based therapies, huge numbers of cells are transplanted at the site of the lesion; most problematic among the risks is their potential neoplastic transformation or contamination of transformable undifferentiated stem cells. Therefore, susceptibility to xenotransplanted tumor cells has to be as high as possible. We examined the transplantability of NOG mice in response to a much lower (10^1) dose of HeLa S3 cells. Unexpectedly, half of the NOG mice (5/10) inoculated with this dosage showed tumor formation in their subcutaneous spaces at 78 days after transplantation. Fig. 1 shows the growth curve of engrafted HeLa S3 cells in NOG mice. The tumors grew progressively and formed a large spheroid mass, although it was localized at the inoculation site and did not invade surrounding areas. A necrotic core was usually observed in advanced tumors (Fig. 2A). Almost all tumor cells, except for the necrotic tumor tissue, were positive for the cell-cycle-regulated nuclear protein, Ki67 antigen, which is widely used as an operational marker of proliferation (Fig. 2B). To determine the origin of the engrafted tumors in the NOG mice, serial sections from formalin-fixed, paraffin-embedded tumors were treated with an anti-HLA monoclonal antibody using the immunoperoxidase staining method. The immunohistochemical reactivity of the anti-HLA monoclonal antibody with the tissue sections demonstrated that the engrafted tumors originated from a human source (Fig. 2C). HeLa S3 cells are immortalized epithelial cells obtained from a human cervical carcinoma (Masters, 2002) and retain the original characteristics of those cells. Moll *et al.* (1982) reported that the antibodies for cytokeratin component 18 strongly stained a variety of tumors of epithelial origin. Therefore, we checked the expression of cytokeratin 8 and 18 intermediate filament proteins (CK8/18) as an epithelial marker. Fig. 2D illustrates the immunohistochemical reactivity of the anti-CK8/18 monoclonal antibody with a tissue section from subcutaneous tumors. Tumor cells were strongly stained, but cells in the stroma were negative. These results demonstrate that the engrafted tumors were progressively growing, originated from human HeLa S3 cells, and were not spontaneously generated by mouse cells.

Manufacturers and regulatory agencies have been developing scientifically based guidelines for the use of cell substrates for biologicals (2006, Center for Biologics Evaluation and Research, Food and Drug Administration (CBER/FDA)). The use of an animal model known to be susceptible to tumor formation by tumorigenic cells has been recommended. Due to their immunodeficiency (T-cell deficient), athymic nude mice have been the animals most commonly used for tumorigenicity testing. In this study, we demonstrated that NOG mice are more susceptible to tumor formation than the nude mice traditionally used. This suggests that NOG mice may be the best choice when identification of a weakly tumorigenic phenotype or a small contamination of transformable undifferentiated cells is important.

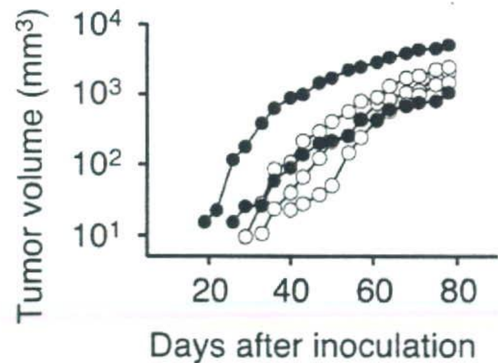

Fig. 1. Growth curve of subcutaneous tumors in NOG mice formed by inoculation with 10^1 HeLa S3 cells. Closed and open circles indicate male (n = 2) and female (n = 3) mice, respectively.

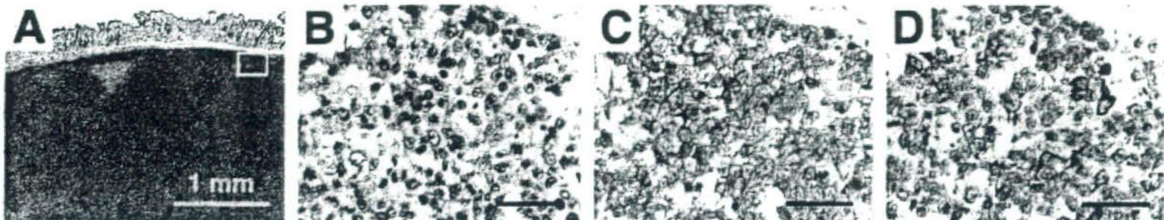

Fig. 2. Histology and immunohistochemistry of subcutaneous tumors in NOG mice formed by inoculation with 10^1 HeLa S3 cells. Serial sections were stained with H&E (A), Ki67 (B), HLA (C) and h-CK8/18 (D). B to D shows the boxed area in A at higher magnification. Scale bar, 50 μm.

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—Articles—

ヒト間葉系幹細胞の *in vitro* 培養期間中の変化について
—*c-myc* をターゲットとした遺伝子発現解析と染色体異常解析—

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Change in Characteristics of Human Mesenchymal Stem Cells during the *In Vitro* Culture
—*c-myc* Gene Expression and Chromosome Aberrations at the *c-myc* locus—

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We investigated mRNA expression of *c-myc* and chromosome aberrations at the *c-myc* locus in the same passage number of human mesenchymal stem cells (hMSCs). To understand the sensitivity of mRNA expression and the induction of chromosome aberrations, we first tested them in hMSC and cancer cell lines (HeLa S3, HOS, and OUMS-27). The *c-myc* mRNA expressions in HeLa S3 and OUMS-27 were significantly higher than those in hMSC, but then those in HOS were not. On the other hand, *c-myc* aberrant cells detected by fluorescence in situ hybridization in HeLa S3, HOS, and OUMS-27 were significantly higher than that in hMSC. Both analyses were performed in hMSCs derived from five donors for the culture period of 50 days. In hMSCs from one donor, the frequency of *c-myc* aberrant cells significantly increased at 20 and 50 days respectively, and each mRNA expressions had a tendency to increase, but there is no significant change among 3, 20 and 50 days. In hMSCs from the others, both endpoints did not change for 50 days. For safe use of somatic stem cells in the regenerative medicine, the investigation of characteristic change of them during the *in vitro* culture is important. In the present study, we showed the mRNA expressions and chromosome aberrations of hMSCs in *in vitro* culture as the first step for establishing of safety evaluation of tissue engineered medical devices using normal hMSCs.

Key words——human mesenchymal stem cells (hMSCs); *c-myc* gene expression; copy number of the *c-myc* locus; (HeLa S3, HOS, OUMS-27)

緒　　言

最近,「人工多能性幹細胞 (iPS 細胞)」と呼ばれる新たな万能細胞がヒトの自己の皮膚細胞から樹立されたことが報告され脚光を浴びている.[1] 今まさにその実用化に向けて国を挙げての取り組みが始まっており, この iPS 細胞の樹立をきっかけに今後益々事故や病気で失った組織の修復, 再建を目指す「再生医療」への期待が高まるであろう. このような背景の中, 成体幹細胞の一種である間葉系幹細胞は, 骨, 軟骨, 筋, 腱, 脂肪, さらには神経細胞や肝細胞, 心筋, 皮膚など胚葉を越えた分化も報告されているため幅広い再生医療分野での利用が期待されている. 現在, 骨髄, 脂肪組織, 臍帯血由来の間葉系幹細胞が, その採取技術及び *in vitro* での培養技術も確立されており, 細胞組織医療機器の材料として現段階で最も実用に近いものの 1 つであると考えられる. しかし, 一般に幹細胞は分化能とともに増殖能を有しており, 人の体内から取り出してある程度の量まで増殖させて再び体内へ移植できるという利点と同時に, 両機能を体内で適切に制御できるかという問題点を含んでいる. 特に細胞組織医薬品や細胞組織医療機器に利用するためには幹細胞を生体内から取り出して *in vitro* で培養して増殖させるという工程を経なければならないため, *in vitro* での培養期間中に幹細胞が目的以外の形質を持った細胞に変化しないこと, 特に遺伝的形質に変化がないことを確認し, 患者に戻される細胞の安全性を担保する方法を確立することは大変重要であろう. 本研究ではその第一歩として, ヒト骨髄由来間葉系幹細

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胞（hMSC）の *in vitro* での培養期間中における細胞の変化について検討する手段として遺伝子発現レベルと染色体レベルでの異常解析とを同じ遺伝子座 *c-myc* で同時に行った．

実験方法

1.　細胞及び培養

ヒト間葉系幹細胞：5 ドナー由来の hMSC（Lonza Walkersville, Inc.）を，Mesenchymal Stem Cell Growth Medium（MSCGM）に Mesenchymal Cell Growth Supplement（MCGS）を加えた培地でそれぞれ培養した．それぞれのドナー情報は Table 1 に示す．

ヒト子宮頸がん由来細胞：HeLa S3（JCRB Cell Bank）は Ham's F-12 Nutrient Mixture［大日本住友製薬㈱］に 10% fetal bovine serum（FBS）を加えた培地で培養した．

ヒト骨肉腫細胞：HOS［大日本住友製薬㈱］は minimum essential medium（MEM; Eagle）に 0.1 mM non-essential amino acid（NEAA）と 10%FBS（Intergen）を加えた培地で培養した．

ヒト軟骨肉腫細胞：OUMS-27（JCRB Cell Bank）は Dulbecco's modified Eagle's medium［DMEM；日水製薬㈱］に 0.1 mM NEAA と 10%FBS を加えた培地で培養した．

2.　Real time（RT）-PCR による mRNA 発現量の定量的解析　　hMSC, HeLa S3, HOS, OUMS-27 から RNeasy Mini Kit（QIAGEN）を用いて total RNA を調製した．抽出した total RNA の cDNA への逆転写は First Strand cDNA Synthesis Kit for RT-PCR（AMV）（Roche Diagnostics）を用いて行った．それぞれの細胞の *c-myc* の mRNA 発現レベルについて Real time RT-PCR 法にて検討した．用いたプライマーは，Forward: 5′-GCGAACACACAACG-TC-3′, Reverse: 5′-CAAGTTCATAGGTGATTGC-T-3′ で，PCR 反応は，95°C で 10 秒，50°C で 15 秒，72°C で 12 秒を 40 サイクル行った．一方，ハウスキーピング遺伝子として Glyceraldehyde 3-phosphate dehydrogenase（GAPDH）の mRNA 発現を検討し，PCR 反応はライトサイクラー専用ヒト mRNA 定量プライマーセット（Search-LC）を用いて行った．定量的解析は，Light Cycler Fast Start DNA Master SYBR Green I（Roche Diagnostics）を用いて Roche Light Cycler（version 4.0）で行った．

3.　染色体数計数と FISH による *c-myc* コピー数異常解析　　hMSC の染色体標本は以下のように作製した．コルセミド（0.02 μg/ml）で一晩処理し，トリプシンで細胞を回収した．75 mM KCl 溶液で室温 20 分間低張処理したのち，カルノア液（氷酢酸：メタノール＝1：3 混液）で 3 回固定した．細胞懸濁液をスライドグラスに滴下し，自然乾燥させた．3 種の腫瘍細胞株の染色体標本は，コルセミド（0.1–0.2 μg/ml）で 2–4 時間処理した以外は，hMSC と同様に作製した．染色体数計数用の標本はギムザ染色し，分裂中期像を撮影して染色体数を計数した．*c-myc* コピー数異常解析は，従来の分染法より簡便に実施できる FISH（fluorescence *in situ* hybridization；蛍光 *in situ* ハイブリダイゼーション）法を採用したが，FISH 解析用標本は窒素ガスを封入したビニル袋に密閉し，−20°C に保管した．

FISH 解析は，*c-myc* の DNA プローブ（Spectrum Orange 標識，VYSIS 社，米国）を用いた．*c-myc* は正常では 8 番染色体の長腕の先端にシグナルが観察されるが，プローブ長が短い（約 120 kb）ため間期細胞でもシグナルの正確な観察ができるという利点がある．

プローブ DNA を 70°C で 5 分間熱変性させ，すぐに氷冷した．染色体 DNA を変性させるために，スライドグラスを 70°C の 70%フォルムアミド溶液に 4 分間浸し，すぐに氷冷した 70%エタノールに移し，その後続けて 85%，100%エタノールに移して自然乾燥させた．スライドグラスに熱変性したプローブ DNA 液を乗せてカバーグラスで覆い，回りをペーパーボンドでシール後，37°C で一晩ハイブリダイズさせた．ハイブリダイズ終了後，45°C の 50%

Table 1.　Donor Information of hMSCs

	Lot No.	Age	Race	Sex
hMSC-A	3F0664	19 Y	African American	F
hMSC-B	4F1560	23 Y	African American	F
hMSC-C	5F0138	19 Y	African American	M
hMSC-D	5F0972	20 Y	African American	M
hMSC-E	4F0218	21 Y	Other[a)]	M

Lonza Walkersville, Inc. *a*) Except for Asian/Oriental, Caucasian, African American, Hispanic and American Indian.

フォルムアミド液で3回，2×sodium saline citrate (SSC) 液で1回，0.1% NP-40を含む2×SSC液で1回洗浄した．その後自然乾燥させて，4′,6-diamidino-2-phenylindole (DAPI) Counterstain (VYSIS社) でマウントし，蛍光顕微鏡 (Nikon E600) で観察した．[2] *c-myc* コピー数異常の観察は，1細胞当たり2点以上のシグナルを持つ間期細胞300個について行い，2シグナルの細胞を正常，3シグナル以上のシグナルを持つ細胞を異常とした．有意差検定にはχ^2検定を用いた．[3]

結　　果

まず，ヒト骨髄由来間葉系幹細胞 (hMSC) と3種類のヒト腫瘍細胞（ヒト子宮頸がん由来細胞：HeLa S3，ヒト骨肉腫細胞：HOS，ヒト軟骨肉腫細胞：OUMS-27）との比較を行った．Fig. 1に染色体数分布を示す．hMSCでの観察では46本にピークを示す分布を示した．腫瘍細胞については，

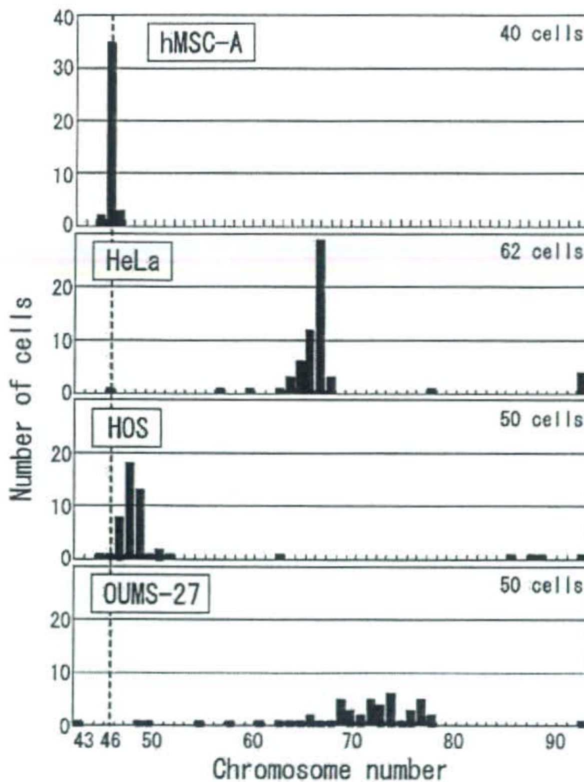

Fig. 1　Distribution of Chromosome Numbers in hMSC-A and Cancer Cells (HeLa S3, HOS, and OUMS-27)

The numbers in the upper-right corner indicate those of metaphases, for which the chromosome number was counted. The normal chromosome number for human is 46 indicated by a dotted line.

HeLa S3細胞及びHOS細胞はそれぞれ67本及び48本にピークを示す分布を示したが，OUMS-27細胞では目立ったピークはなく，69–78本を中心として幅広い分布を示した．46本にピークを持つ正常な染色体数分布を示したhMSCに対し，3種類の腫瘍細胞はそれぞれの染色体数分布に大きな違いがみられ，すべて正常とは異なるものであった．Figure 2に*c-myc*のmRNA発現レベルと染色体異常率を示す．mRNA発現レベルをGAPDHの相対値として示し，染色体異常については300細胞中に観察された異常細胞数から求めた異常細胞率を示した．*c-myc*のmRNA発現レベル [Fig. 2(A)] は，hMSCに比べてHeLa S3及びOUMS-27では有意にその発現が高かったものの，HOSでは有意な差は認められなかった（*t*-検定)．一方，染色体レベルでの異常細胞率 [Fig. 2(B)] は，HeLa S3及びOUMS-27においてはどちらも100%近い異常率を示し，HOSでは15%程度の異常細胞率であったが，異常細胞率が5%以下のhMSCと比較すると3種類すべての腫瘍細胞株で有意に高い異常率を示した (χ^2検定).

次に，hMSCの*in vitro*での培養期間中における遺伝子発現レベルの変化と*c-myc*コピー数異常解析を行った．まず5ドナー由来のhMSC (hMSC-A, B, C, D, E) について，培養期間160日程度までの増殖曲線をFig. 3に示した．どの細胞も培養50日間くらいまでは比較的よく増殖しているが，この時点で総細胞数がドナー間で既に最大100倍以上も違っていた．そしてその後はどの細胞も増殖速度が徐々に低下してきた．増殖速度についてはそれぞれのドナー間で差がみられたが，今回用いたhMSCはどれもLonza Walkersville社より購入したもので，その細胞調製方法は一定であると思われるため，骨髄を採取されたそれぞれの個人差が大きく影響するものと考えられる．

実際に幹細胞を細胞組織医療機器や細胞治療の材料として用いる臨床研究の場合，生体内から取り出して*in vitro*で継代培養する期間はだいたい1ヵ月以内が多い．そこで，実際に細胞組織医薬品・医療機器等の材料として間葉系幹細胞を用いる場合を想定し，妥当な培養期間内（多少長めに設定し50日以内）で*c-myc*の変化について検討した．5ドナー由来のhMSC (hMSC-A, B, C, D, E) を用いて，*c-*

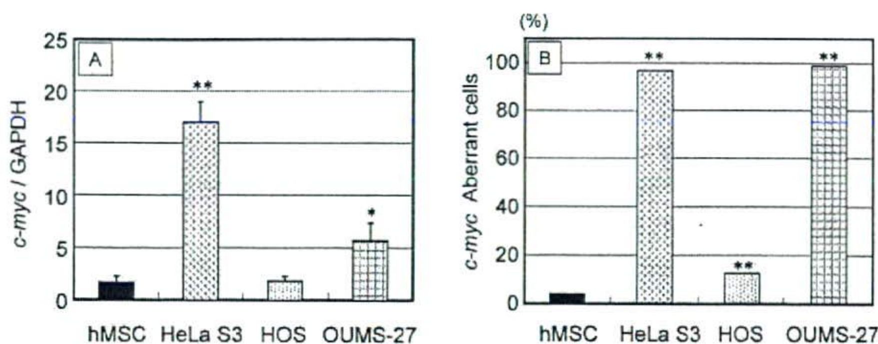

Fig. 2 *c-myc* Gene Expression (A) and Chromosome Aberrations at the *c-myc* Locus (B) in hMSC and Cancer Cells (HeLa S3, HOS, and OUMS-27)

(A) The relative expressions of *c-myc* to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in hMSC, HeLa S3, HOS, and OUMS-27 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 hMSC (**$p<0.01$, *$p<0.05$). (B) Frequency (%) of *c-myc* aberrant cells in hMSC, HeLa S3, HOS, and OUMS-27. 300 interphase cells were analyzed after hybridization with the *c-myc* probe. **$p<0.01$ by a chi-square test.

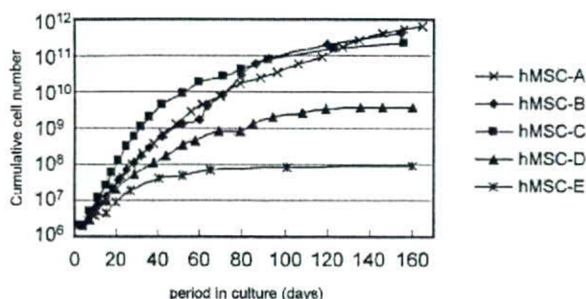

Fig. 3 Proliferation of hMSCs

hMSCs were seeded at a density of 6000 cells/cm^2 and when they were just subconfluent they were subcultured and then counted the cell numbers.

myc の mRNA 発現レベルと染色体レベルでの変化について検討した結果を Fig. 4 に示す．hMSC-A, B, C, D では，培養期間 50 日以内における *c-myc* の mRNA 発現レベルとコピー数には，有意な変化は認められなかった．しかし，hMSC-E においては，*c-myc* コピー数異常細胞率が培養 20 日間で 7.7 %，50 日間で 13.7% と培養日数の経過に伴い増加し，3 日間（3.3%）に比べていずれも有意な増加がみられた．一方，mRNA レベルについては培養 20 日間及び 50 日間で 3 日間に比べて平均値では 2 倍程度の増加傾向がみられたものの，いずれも統計学的に有意な差は認められなかった．また，hMSC-A は 50 日以降も培養を続け，ほとんど増殖しなくなり細胞老化が認められる状態[4]まで観察した．培養期間 152 日間の細胞における *c-myc* FISH 解析によるコピー数異常細胞率を測定した結果，異常細胞率が 15.7% であり 3 日間（5.0%）に比べて有意な増加が認められた．

京都大学で樹立された国産初のヒト ES 細胞株で同様に解析した *c-myc* コピー数異常細胞率を Fig. 5 に示す．京都大学再生医科学研究所末盛博文先生に培養期間の異なる 3 種のヒト ES 細胞株染色体標本を提供して頂いた．KhES-2 の 3 ヵ月培養標本では，その培養開始時よりも有意な *c-myc* コピー数異常細胞の増加が観察されたが，さらに培養を続けた 12 ヵ月目の標本では有意差は認められなかった．

考 察

c-myc は，細胞分裂を促進するアクセル役となる「がん遺伝子」の中で最もよく知られた遺伝子の 1 つで，多くのがんに関係していることが分かっている．また細胞の老化や不死化に係わるテロメレースを活性化する転写因子であり，細胞周期にも深く係わる．さらに，昨今話題の iPS 細胞作製の際に導入される 4 つの遺伝子のうちの 1 つでもあり，その後「がん遺伝子」である *c-myc* を除いた 3 遺伝子の導入によっても iPS 細胞が作製可能であることが報告されている.[5] 本研究の最終的な目標が幹細胞の *in vitro* での継代培養中の安全性を担保できる方法の確立であり，幹細胞自身が増殖能を持つことからその最も異常な変化として「がん化」が懸念されているため，本研究では細胞増殖やがんに係わる遺伝子 *c-myc* に着目し検討した．

「再生医療」の早期実現が待望されている今，その材料の 1 つとして間葉系幹細胞の有用性が期待されている．幹細胞の大きな特徴としては，様々な組

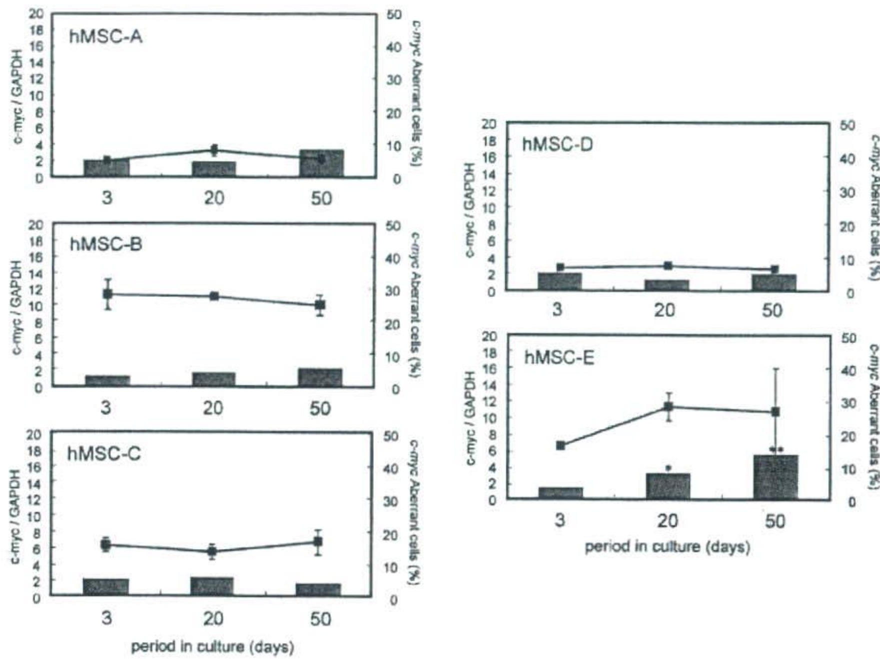

Fig. 4 Change in *c-myc* Gene Expression and Chromosome Aberrations at the *c-myc* Locus of hMSC-A, B, C, D, E during the *in vitro* Culture

Expressions of *c-myc* relative to GAPDH were investigated by quantitative RT-PCR. Mean values with SDs from three independent experiments are presented in the line graph. Frequency (%) of *c-myc* aberrant cells is presented in the bar graph. 300 interphase cells were analyzed after hybridization with the *c-myc* probe. $^{**}p<0.01$, $^{*}p<0.05$ by a chi-square test.

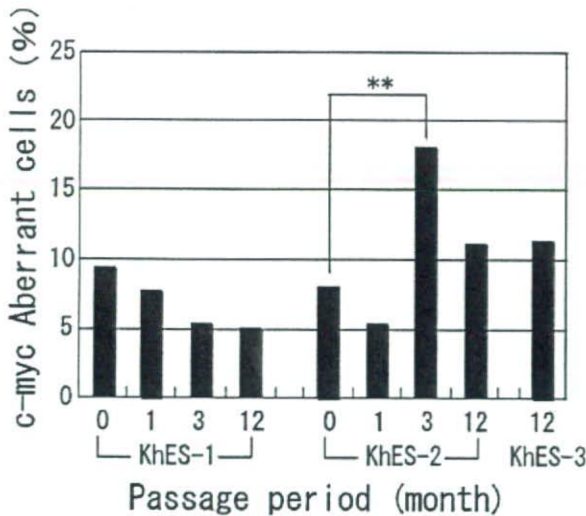

Fig. 5 Frequency of *c-myc* Aberrant Cells in the Three Human ES Cell Lines Supplied by Dr. Suemori (Kyoto University) as Chromosome Preparations

300 interphase cells were analyzed after hybridization with the *c-myc* probe. $^{**}p<0.01$.

織への多分化能を保ちながら増殖する点であり，実際に臨床において幹細胞を利用する場合には，幹細胞を体内から取り出したのち *in vitro* で培養して増殖させなければならない．その培養過程において万が一細胞に不都合な変化が起きてしまった場合，その後患者の体内に戻した際の安全性は担保できない．そのため，幹細胞の *in vitro* 培養中の変化の観察は重要であると考え，本研究では観察の手段として2通りの方法（遺伝子レベルでの発現とコピー数異常）を用いて行い，同じ遺伝子座 *c-myc* について同時に検討した．

まず，幹細胞と腫瘍細胞を用いて *c-myc* の mRNA 発現レベルとコピー数異常について検討したところ，その結果には違いがみられた（Fig. 2）．HeLa S3 及び OUMS-27 のように染色体レベルでの異常細胞率が 100％近い腫瘍細胞については，mRNA 発現レベルも幹細胞に比べて有意に高い値を示したが，異常細胞率が 15％程度であった HOS では，mRNA 発現レベルに幹細胞（hMSC）との有意な差は認められなかった．さらに，実際に幹細胞の臨床現場での利用を想定した培養期間における経時的変化についても *c-myc* の mRNA レベルとコピー数での検討結果には違いがみられた（Fig. 4）．用いた5ドナー由来の hMSC（hMSC-A，B，C，D，

E）すべてにおいて，*c-myc* の mRNA 発現レベルには有意な変化は認められなかったが，コピー数異常細胞率については 5 ドナー中の 1 ドナー（hMSC-E）において有意な増加（～13.7%）が認められた．以上の結果から，染色体レベルでの異常頻度が非常に高い細胞ではその mRNA 発現レベルにも有意な差が現れてくるが，異常細胞率が 15% 程度（つまり正常細胞が 85% 程度と数としては異常細胞よりもかなり多く含まれている状態）の細胞では，mRNA 発現レベルにおいて有意な変化を検出できない可能性を示している．このことから，hMSC をはじめ培養細胞の増殖異常の指標となる *c-myc* 遺伝子座に関しては，遺伝子発現の検討だけでなくコピー数の検討を行う必要があると考えられた．また，*c-myc* 以外の領域についても同様の検討を行っていく必要もあるであろう．

本研究では，幹細胞の *in vitro* 培養期間中の変化について観察するためのよりよい評価法を探るための第一歩として *c-myc* を用いて mRNA 発現レベルとコピー数での 2 つの方法で検討した結果を示した．「再生医療」を目指した臨床応用に向けて，細胞の培養条件や目的による利用細胞の選び方なども考慮していくことが重要であろう．[6]

謝辞 国産初のヒト ES 細胞標本を提供して頂いた京都大学再生医科学研究所中辻憲夫教授，末盛博文准教授に感謝申し上げます．本研究は，創薬等ヒューマンサイエンス総合研究事業「幹細胞等を用いた細胞組織医療機器の開発と評価技術の標準化」及び再生医療事業「感染リスクの排除，同一性の確保，がん化等の抑制及び培地等による有害作用の防止に関する研究」にて実施された研究の一部である．

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The Metabolism and Distribution of Docosapentaenoic Acid (n-6) in the Liver and Testis of Growing Rats

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To investigate the metabolism and distribution of docosapentaenoic acid (22:5n-6, DPA) in the liver and testis of growing rats, 22:5n-6 was administered to their dams. Newborn rats with a low hepatic arachidonic acid (20:4n-6, AA) level were generated by administrating a diet rich in docosahexaenoic acid (22:6n-3, DHA) but n-6 fatty acid (FA) free to pregnant dams. After parturition, 22:5n-6 or linoleic acid (18:2n-6, LA) was administered with a high level of 22:6n-3 to the dams until weaning. At weaning, the hepatic 20:4n-6 level was significantly highest in the DPA-DHA but not LA-DHA diet-fed animals. The hepatic delta-6 desaturase (D6D) mRNA abundance was significantly lower in both the LA-DHA and DPA-DHA diet-fed animals, connoted with the 20:4n-6 content recovered by 22:5n-6 that did not involve D6D and supporting the occurrence of retroconversion in the liver of the growing rats. The low D6D level in the 3-week-old testis was not in proportion to the elevated 22:5n-6 level, implying that early testicular 22:5n-6 accumulation might require supply from the circulation system.

Key words: docosapentaenoic acid; docosahexaenoic acid; arachidonic acid; delta-6 desaturase; retroconversion

Docosahexaenoic acid (22:6n-3, DHA), the most abundant n-3 fatty acid normally present in human and animal tissues, has specific functions in the brain and retina.[1–4] It has been reported that infant formula containing 22:6n-3 was associated with improved growth and development.[5,6] Moreover, formula containing 22:6n-3 has been shown to enable preterm infants to achieve immune development similar to that seen with breast-milk feeding.[7] Arachidonic acid (20:4n-6, AA), which is important in cell membrane phospholipids and serves as the precursor of eicosanoids, is maintained at a constant level under normal conditions *in vivo*. It has been shown that breast milk containing 20:4n-6 contributed to infant development.[8] There is also report that 20:4n-6 deficiency in preterm infants may lead to impaired growth over the first year of life.[9] In studies on 22:6n-3 supplementation, however, a high dietary intake of 22:6n-3 resulted not only in an increased 22:6n-3 content, but also in a drastically decreased 20:4n-6 content.[10,11] This decrease in 20:4n-6 has been proposed to be related to the inhibition of delta-6 desaturase (D6D), reducing the synthesis of 20:4n-6 from linoleic acid (18:2n-6, LA). Both 22:6n-3 and 20:4n-6 are essential components for infant growth and development, and a lack of either may lead to impaired growth. Therefore, the 20:4n-6 decline caused by 22:6n-3 still requires a solution.

The docosapentaenoic acid (22:5n-6, DPA) content in most organisms is low, the n-3 isomer of 22:5n-6 in most fish oils being at a higher level than the n-6 isomer.[12] It is also known that 22:5n-6 is β-oxidized to 20:4n-6,[13–16] and that a deficiency of n-3 essential fatty acids in animals causes a compensatory rise in the 22:5n-6 level in the brain/retina,[17,18] although the physiological function of 22:5n-6 has not yet been clarified. In our previous *in vivo* and *in vitro* study, 22:5n-6 was found to blunt the decrease in 20:4n-6 level, but 18:2n-6 could not prevent the same decline in 20:4n-6 caused by a high 22:6n-3 administration level, implying that the 20:4n-6 level increased by 22:5n-6 supplementation was not affected by 22:6n-3.[19]

There has been no previous report on the effect of 22:6n-3 on the 20:4n-6 level from pregnancy; studies on

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Abbreviations: D6D, delta-6 desaturase; D5D, delta-5 desaturase; FA, fatty acid; PUFA, polyunsaturated fatty acid; GC, gas-chromatography

the effect of 22:5n-6 and 18:2n-6 on the biosynthesis of 20:4n-6 conditioned by high 22:6n-3 supplementation are also limited. It would therefore be valuable to compare the effects of 22:5n-6 and 18:2n-6, the two possible sources of 20:4n-6 accumulation, under a high 22:6n-3 administration level on the accumulation of 20:4n-6 in pups from pregnancy to weaning.

A second objective would be to clarify the source of the testicular accumulation of 22:5n-6 during growth, because it has been found that 22:5n-6 was present in a high concentration specifically in rat testis, where the level of 22:5n-6 has been found to be independent of the kind and quantity of oil in the diet.[19] Therefore, the testicular 22:5n-6 accumulation was investigated in newborn and weaned rats to clarify the source of 22:5n-6 in the testis during growth.

Materials and Methods

Materials. The DPA-DHA oil prepared from a single-cell marine microbe was presented by Suntory (Osaka, Japan). Safflower oil, rapeseed oil and soybean oil were presented by The Nissin Oillio Group (Tokyo, Japan). DHA-35G oil was presented by Japan Scientific Feeds Association. All other chemicals and reagents were of analytical grade.

Animals and diets. All procedures for the use and care of the animals for laboratory research were approved by the Animal Experiment Ethics Committee of Ochanomizu University. Eleven-week-old Wistar rats were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). All the rats were initially fed on a commercial diet (CE-2) from Nippon Clea Co. (Tokyo, Japan) for one week. The animals were housed individually in an air-conditioned room at 23 ± 1 °C with a 12 h light-dark cycle and were given the experimental diets and water *ad libitum*.

The test schedule was shown in Fig. 1. After coitus, the dams were fed with DHA-rich oil (DHA group: n-6 FA-free diet) or safflower oil (SO group: n-6 FA-rich control diet) until parturition to generate newborn rats with low hepatic 20:4n-6 content. At birth, the dams were subdivided into the LA-DHA group (LA, 26%) and DPA-DHA group (LA, 17%; DPA, 7.8%) with no difference in body weight (data not shown). Before breast feeding, newborn pups with similar body weight were randomly selected from the DPA-DHA and LA-DHA groups to provide 5–10 samples for an analysis of the FA composition and D6D mRNA expression level at birth (week 0). The dams in the SO group continued to receive the same diet. The newborn pups were breast-fed for 3 weeks until weaning.

All the diets were prepared by mixing 5% (w/w) of the experimental oil mixtures with the basal diet. The basal diet, which was prepared according to the standards given by the American Institute of Nutrition (AIN),[20] was provided by Eisai Co., and contained the following percentage of ingredients according to weight: casein, 20; glucose, 25; sucrose, 25; cornstarch, 15; filter paper, 5; mineral mixture (AIN-76), 3.5; vitamin mixture (AIN-76), 1; choline bitartrate, 0.2; and DL-methionine, 0.3. The oil mixtures were prepared as follows: (i) SO diet, safflower oil; (ii) DHA diet, DHA-35G oil; (iii) DPA-DHA diet, DPA-DHA oil/safflower oil, 4:1; (iv) LA-DHA diet, (Soybean oil/rapeseed oil, 1:1)/DHA ethyl ester(65:35). The total amounts of n-6 and n-3 fatty acids in the dams' diets were adjusted to similar levels (Table 1).

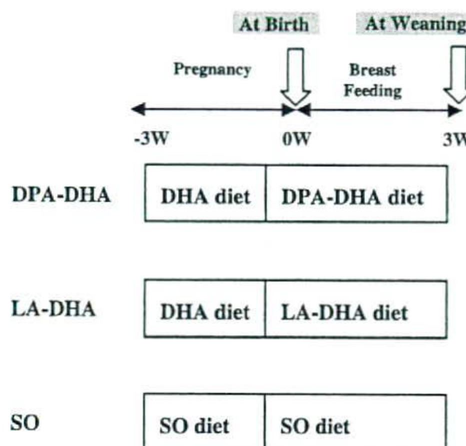

Fig. 1. Experimental Design and Diets Given to the Dams.
SO, safflower oil diet; LA-DHA, 18:2n-6-rich diet; DPA-DHA, 22:5n-6-rich diet; −3 W, three weeks before parturition; 0 W, at parturition; 3 W, at weaning. All the experimental diets were administered to dams. Analyses were conducted at the birth (week 0) and weaning (week 3) stages.

Table 1. Fatty Acid Composition of the Diets (%)

	SO	DHA	LA-DHA	DPA-DHA
16:0	8.8	20.1	7.0	40.7
18:0	3.3	4.4	2.9	2.2
18:1(n-9)	16.2	21.2	31.9	4.4
18:2(n-6)	70.9	3.0	26.0	17.3
20:4(n-6)	0.0	0.0	0.0	0.0
22:5(n-6)	0.0	0.0	0.0	7.8
18:3(n-3)	0.9	0.0	5.4	0.0
20:5(n-3)	0.0	10.8	0.0	0.0
22:6(n-3)	0.0	40.4	26.9	28.8

Each Value is expressed as a percentage of weight. SO, safflower oil diet; DHA, DHA-rich diet; LA-DHA, 18:2n-6-rich diet; DPA-DHA, 22:5n-6-rich diet.

Tissues collection. All animals were killed by blood collection from the abdominal aorta. Collected tissues were washed by saline and stored at −80 °C.

Lipid analyses. Lipids were extracted from tissues of the rats by the method of Folch *et al.*[21] Each lipid extract was methyl esterified with HCl/methanol by the method mentioned in our previous paper to measure the composition of fatty acids in the tissue.[22] The fatty acid

methyl esters were measured by gas-liquid chromatography (Perkin Elmer AutoSystemGC; Palo Alto, CA, USA) with a Rascot Silliar 5CP capillary column (0.25 mm × 50 m; Nihon Chromato Works, Tokyo, Japan) under the conditions previously described in detail.[22)]

Real-time PCR analysis. Total RNA was extracted from the tissues by the acid guanidinium-phenol-chloroform method.[23)] The mRNA expression level of D6D was determined by the real time PCR method.

The Primer design of D6D was based on the sequences obtained from GenBank and the location of the open reading frame as determined with TaqMan Applied Biosystems Primer Express 1.0 software. The forward and reverse primers used were as follows: (5′-GTTCTTCTTTCTCCTCCTGTCCC-3′) and (5′CA-TTGCCGAAGTACGAGAGGAT-3′). Each sample was subjected to quantitative real-time PCR, using the GeneAmp 5700 sequence detection system (Applied Biosystems).

Statistical analysis. Each result is shown as the mean ± SD. The significance of differences was evaluated by analysis of variance (ANOVA) and the Bonferroni-Dunn post-hoc test, or by Student's *t*-test. Analyses were performed by using the StatView (System 4.02) computer package or Microsoft Excel software.

Results

Growth parameters and tissue weights

No significant differences in the food intake and body weight gain were apparent among the animal groups (data not shown). We conclude that the administration of the various dietary PUFAs had no adverse influence on the growth of the rats.

Effect on the fatty acid composition in the liver of pups

Pregnant dams were fed by the DHA-rich diet for three weeks until parturition, and the hepatic fatty acid composition of the pups was investigated at birth (week 0) (Table 2). The 22:6n-3 content in the DHA diet was incorporated into the newborn liver of the DHA group feeding dams with the DHA diet containing only 3% of 18:2n-6 as the n-6 fatty acid successfully in created a newborn liver with a significantly low level of 20:4n-6 compared to the SO group. The other major n-6 FAs were at a very low level (18:2n-6) or not detected (22:4n-6 and 22:5n-6) in the newborn liver of the DHA group.

The low 20:4n-6 environment created at birth provided an appropriate platform to compare the efficiency of 18:2n-6 and 22:5n-6, the two possible sources of 20:4n-6 accumulation. 18:2n-6 and 22:5n-6 were respectively supplemented with 22:6n-3 in the diet for the dams to feed the LA-DHA and DPA-DHA groups. To confirm whether the various dietary PUFAs had been incorporated into the milk of the dams in each group, the milk was collected at weaning (week 3) for a fatty acid analysis (Table 3). 18:2n-6, 22:5n-6 and 22:6n-3, which were the major PUFAs in the dams' diet, were found to have been incorporated into the milk. The fatty acid profile in the liver at weaning (Week 3) is shown in Table 4. Although fed by dams whose diet had been supplemented with a high 22:6n-3 content, 18:2n-6 and 22:5n-6 was respectively incorporated into the liver of the LA-DHA and DPA-DHA pups. The 18:2n-6 level was significantly higher in the LA-DHA group, and the 22:5n-6 level was significantly higher in the DPA-DHA group. The level of 20:4n-6 in the liver of the weaned DPA-DHA group was significantly higher than that of the LA-DHA group. Comparing the 20:4n-6 levels in the livers of growing rats in the LA-DHA and DPA-DHA groups, 22:5n-6 was found to be more effective in its contribution to 20:4n-6 accumulation when administered with a significant amount of 22:6n-3.

Effect on the D6D mRNA expression level in the liver of the pups

D6D, the rate-determining enzyme in both the n-3 and n-6 pathway, was investigated in the liver of the newborn rats (week 0) (Fig. 2). The mRNA expression level of D6D was significantly low in the DHA group

Table 2. Fatty Acid Composition in the Liver at Birth (Week 0)

	SO	DHA
18:0	15.1 ± 0.47^a	12.8 ± 0.23^b
18:1n-9	5.87 ± 0.63	4.76 ± 0.37
18:2n-6	13.2 ± 1.25^a	2.70 ± 0.13^b
20:4n-6	13.6 ± 0.71^a	4.99 ± 0.29^b
22:4n-6	1.78 ± 0.11	ND
22:5n-6	2.45 ± 0.14	ND
20:5n-3	ND	0.90 ± 0.17
22:6n-3	4.99 ± 0.34^a	7.88 ± 0.38^b

Dams were fed on the experimental diets for 3 weeks until parturition. The FA composition in newborn liver was analyzed by GC. Each result is expressed in nmol/g of tissue by the mean ± SD (n = 5–10). SO, safflower oil diet; DHA, DHA-rich diet. The significance of differences between different dietary treatments was analyzed by Student's *t-test*. Bars not sharing a common roman letter are significantly different ($p < 0.05$).

Table 3. Fatty Acid Composition of the Milk (%)

	SO	LA-DHA	DPA-DHA
16:0	20.7	22.7	24.3
18:0	2.9	2.7	3.9
18:1(n-9)	13.1	12.4	9.8
18:2(n-6)	17.0	6.7	6.4
20:4(n-6)	0.6	0.0	0.4
22:5(n-6)	0.0	0.0	1.2
18:3(n-3)	0.2	1.1	0.0
20:5(n-3)	0.0	0.3	0.0
22:6(n-3)	0.0	2.8	6.1

Each value is expressed as a percentage of weight. SO, safflower oil diet; LA-DHA, 18:2n-6-rich diet; DPA-DHA, 22:5n-6-rich diet.

Table 4. Fatty Acid Composition in the Liver at Weaning (Week 3)

	SO	LA-DHA	DPA-DHA
18:0	16.7 ± 0.92	17.4 ± 0.37	16.8 ± 0.42
18:1n-9	6.87 ± 0.78[a]	4.55 ± 0.24[b]	4.04 ± 0.19[b]
18:2n-6	18.7 ± 1.88[a]	7.69 ± 0.17[b]	5.93 ± 0.21[b]
20:4n-6	16.8 ± 1.08[a]	5.74 ± 0.21[b]	8.44 ± 0.45[c]
22:4n-6	1.73 ± 0.16	ND	ND
22:5n-6	2.93 ± 0.14[a]	ND	2.09 ± 0.20[b]
20:5n-3	ND	2.20 ± 0.12[a]	0.83 ± 0.09[b]
22:6n-3	3.84 ± 0.30[a]	22.8 ± 1.68[b]	22.8 ± 1.43[b]

Dams were fed on the experimental diets for 3 weeks until weaning. The FA composition in the weaned liver was analyzed by GC. Each Result is expressed in nmol/g of tissue by the mean ± SD (n = 3–6). SO, safflower oil diet; LA-DHA, 18:2n-6-rich diet; DPA-DHA, 22:5n-6-rich diet. The significance of differences between different dietary treatments was evaluated by an analysis of variance (ANOVA) and the Bonferroni-Dunn post-hoc test. Bars not sharing a common roman letter are significantly different ($p < 0.05$).

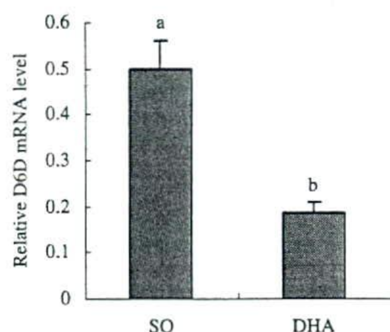

Fig. 2. Effect on D6D in the Liver of Newborn Rats (Week 0).

The mRNA expression level of D6D was measured by real-time PCR. Each result is expressed as the relative concentration (n = 5–10). SO, safflower oil diet; DHA, 22:6n-3-rich diet. The significance of differences between different dietary treatments was analyzed by Student's *t-test*. Bars not sharing a common roman letter are significantly different ($p < 0.05$).

liver. The D6D mRNA abundance in the weaned rats at week 3 is shown in Fig. 3. The D6D mRNA levels were very low in both the LA-DHA and DPA-DHA group weaned liver, there being no significant difference between the D6D mRNA levels in both these groups.

Effect on the fatty acid composition in the testis of the pups

The fatty acid composition in newborn testis at week 0 is shown in Table 5. 22:6n-3, which was the major PUFA included in the DHA diet, was found to be significantly high in DHA group testis. No significant difference was found between the levels of 20:4n-6 and 22:5n-6, the major n-6 metabolites in the testis.

The testicular fatty acid composition at week 3 is shown in Table 6. 22:6n-3 was significantly high in the testis of the weaned LA-DHA and DPA-DHA groups, reflecting its effect from the milk of the dams fed with LA-DHA or DPA-DHA diets. 22:5n-6 in the testis of the weaned DPA-DHA group was significantly higher than that of the LA-DHA group.

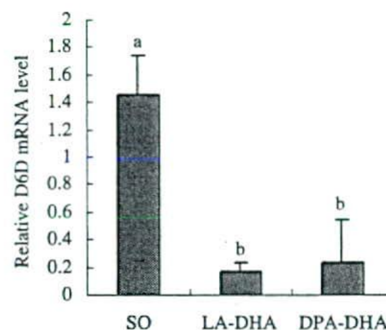

Fig. 3. Effect on D6D in the Liver of Weaned Rats (Week 3).

The mRNA expression level of D6D was measured by real-time PCR. Each result is expressed as the relative concentration (n = 3–6). SO, safflower oil diet; LA-DHA, 18:2n-6-rich diet; DPA-DHA, 22:5n-6-rich diet. The significance of differences between different dietary treatments was evaluated by an analysis of variance (ANOVA) and the Bonferroni-Dunn post-hoc test. Bars not sharing a common roman letter are significantly different ($p < 0.05$).

Table 5. The Fatty Acid Composition in the Testis at Birth (Week 0)

	SO	DHA
18:0	10.1 ± 1.97[a]	17.5 ± 2.54[b]
18:1n-9	4.13 ± 0.27	5.65 ± 1.10
18:2n-6	1.32 ± 0.19[a]	2.19 ± 0.40[b]
20:4n-6	2.93 ± 0.23	2.59 ± 0.21
22:4n-6	0.61 ± 0.02	ND
22:5n-6	0.34 ± 0.01	0.51 ± 0.10
20:5n-3	ND	0.37 ± 0.09
22:6n-3	0.65 ± 0.03[a]	1.78 ± 0.02[b]

Dams were fed on the experimental diets for 3 weeks until parturition. The FA composition in the newborn testis was analyzed by GC. Each result is expressed in nmol/g of tissue by the mean ± SD (n = 5–10). SO, safflower oil diet; DHA, 22:6n-3-rich diet. The significance of differences between different dietary treatments was analyzed by Student's *t-test*. Bars not sharing a common roman letter are significantly different ($p < 0.05$).

Table 6. Fatty Acid Composition in the Testis at Weaning (Week 3)

	SO	LA-DHA	DPA-DHA
18:0	5.43 ± 0.08	5.05 ± 0.31	5.61 ± 0.19
18:1n-9	4.85 ± 0.10[a]	5.94 ± 0.51[b]	4.40 ± 0.23[a]
18:2n-6	1.40 ± 0.04[a]	1.49 ± 0.15[a]	0.95 ± 0.05[b]
20:4n-6	5.45 ± 0.22[a]	3.32 ± 0.39[b]	3.52 ± 0.25[b]
22:4n-6	0.78 ± 0.02[a]	0.19 ± 0.03[b]	0.26 ± 0.02[b]
22:5n-6	3.27 ± 0.26[a]	0.18 ± 0.02[b]	0.63 ± 0.07[c]
20:5n-3	ND	0.26 ± 0.03[a]	0.11 ± 0.01[b]
22:6n-3	0.64 ± 0.02[a]	3.24 ± 0.47[b]	2.56 ± 0.24[b]

Dams were fed on the experimental diets for 3 weeks until weaning. The FA composition in weaned testis was analyzed by GC. Each result is expressed in nmol/g of tissue by the mean ± SD (n = 3–6). SO, safflower oil diet; LA-DHA, 18:2n-6-rich diet; DPA-DHA, 22:5n-6-rich diet. The significance of differences between different dietary treatments was evaluated by an analysis of variance (ANOVA) and the Bonferroni-Dunn post-hoc test. Bars not sharing a common roman letter are significantly different ($p < 0.05$).

Effect on the D6D mRNA expression level in the testis of the pups

The testicular D6D mRNA expression could not be investigated at week 0 due to the limited size of the

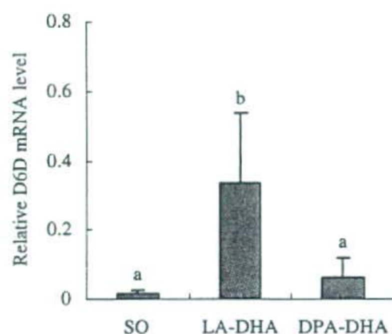

Fig. 4. Effect on D6D in the Testis of Weaned Rats (Week 3).
The mRNA expression level of D6D was measured by real-time PCR. Each Result is expressed as the relative concentration (n = 3–6). SO, safflower oil diet; LA-DHA, 18:2n-6-rich diet; DPA-DHA, 22:5n-6-rich diet. The significance of differences between different dietary treatments was evaluated by an analysis of variance (ANOVA) and the Bonferroni-Dunn post-hoc test. Bars not sharing a common roman letter are significantly different ($p < 0.05$).

newborn organs. The results for the testis of the weaned pups at week 3 are shown in Fig. 4. The D6D mRNA level was significantly higher in the testis of the LA-DHA group than of the DPA-DHA group. There was no significant difference between the D6D mRNA level in the DPA-DHA and SO groups. Focusing on the results for the SO group, the low D6D expression level was not in proportion to the increased accumulation of major n-6 metabolites, especially 22:5n-6, in the testis of the SO group.

Discussion

Feeding pregnant dams with a n-6 FA-free diet in the present study successfully created newborn pups with a significantly low level of 20:4n-6 in the liver of the DHA group which provided a favorable environment to compare the effect of the two possible sources for 20:4n-6 synthesis: 18:2n-6 and 22:5n-6. After 3 weeks of diet intervention with the dams fed the LA-DHA diet, the 20:4n-6 level in their pups was unchanged from the 20:4n-6 level at birth (Tables 3 and 4). However, intervention with 22:5n-6 in the dams fed with the DPA-DHA diet resulted in the hepatic 20:4n-6 level in their pups being increased by 1.5-fold, showing that 20:4n-6 synthesis by 22:5n-6 was more efficient than by 18:2n-6. Moreover, DHA diet intervention in the dams resulted in the decline of D6D mRNA expression in both the newborn and weaned liver of their pups (Figs. 2 and 3). This D6D decline did not stop the increase of 20:4n-6 by 22:5n-6, which implies that the conversion of 22:5n-6 to 20:4n-6 did not involve D6D and supports the retroconversion of 22:5n-6 to 20:4n-6 that had been reported in our previous study.[19] Studies on the retroconversion of 22:5n-6 to 20:4n-6 in rat liver have reported that the retroconversion occured with 22:4n-6 as the intermediate, suggesting that retroconversion took place by direct hydrogenation and subsequent β-oxidation.[24]

It has been well reported that a 20:4n-6 decline would be caused by 22:6n-3 administration *in vivo*.[25–28] In the present study, the hepatic 20:4n-6 decline in newborn rats resulted from indirect diet intervention by supplementing the experimental diets fed to the dams. The D6D mRNA expression level showed that D6D was significantly low in the newborn pups fed by the dams that had received the DHA diet, which may account for the decline of 20:4n-6 in the newborn liver. As D6D is the major enzyme catalyzing the rate-determining first step of the n-6 and n-3 PUFA metabolic pathway, which involves the conversion of linoleic acid (LA, 18:2n-6) to γ-linolenic acid (GLA, 18:3n-6) and of ALA to 18:4n-3, the metabolism of 18:2n-6 cannot start without D6D. There have been studies reporting the inhibitory effect of PUFAs on the expression of D5D and D6D in the liver through the regulation of sterol-regulatory element binding protein 1 (SREBP-1) at the transcription level.[29,30] Moreover, it has been reported that the expression level of D5D and D6D was positively regulated by SREBP-1 in the liver as a result of the presence of a sterol-regulatory element in the promoter region of D6D and probably D5D.[31,32] According to these reports, the low D6D expression level caused by PUFAs in this study was probably related to the suppression of SREBP-1 at the transcription level.

20:4n-6 is an essential component of early growth.[8,9] Therefore, the abnormal decline of 20:4n-6 caused by high 22:6n-3 supplementation still requires a solution. Our former and present studies provided animal *in vivo* and *in vitro* data demonstrating that the retroconversion of 22:5n-6 to 20:4n-6 was independent of a high 22:6n-3 administration; this may suggest a solution to blunt the abnormal 20:4n-6 decline caused by a high 22:6n-3 administration by applying 22:5n-6 to 22:6n-3. A human clinical study has reported the effect on cardiovascular risk factors of a marine oil rich in 22:6n-3 and 22:5n-6, similar to the oil used in this study, showing that the oil was safe and did not adversely affect cardiovascular disease.[33] In future, human data on the retroconversion of 22:5n-6 to 20:4n-6 caused by a high 22:6n-3 administration will be required.

In our previous study, a high 22:5n-6 concentration was unique to the rat testis, where the level of 22:5n-6 was found to be independent of the quality and quantity of oil supplemented to the diet for mature rats.[19] The testis is an organ that consumes C22 PUFA to transport the sperm into the vesiculae seminalis.[34] At homeostasis, 22:5n-6 as the dominant C22 PUFA in the testis is believed to be involved in the function of sperm transportation.[34] A study on the testicular development of rats maintained from weaning age to 63 weeks has reported that rats fed with a n-6 FA deficient diet suffered testicular degeneration with the loss of germinal cells and the 22:5n-6 level was being markedly low in the testis.[35] In this study, the fatty acid profile in the testis of newborn and weaned rats showed that 22:5n-6

in the testes was at a level independent of the dietary effect. These results support the unique presence and importance of 22:5n-6 during the development of this organ (Tables 5 and 6).

It is known that testicular lipids have an active metabolism, arising from both dietary sources and from the processes of synthesis, elongation, desaturation, interconversion, esterification and oxidation by the testicular tissue itself,[36] although the source of 22:5n-6 accumulation during early growth has not yet been clarified. In the present study, focusing on the results for the SO group testis at week 3, the low D6D expression level was not in proportion to the increased accumulation of major n-6 metabolites, especially 22:5n-6 (Table 6). This suggests that the accumulation of 22:5n-6 in the testis at the growing stage was not only supported by local production, but also by uptake from the circulation system, which was probably provided by synthesis in the liver. According to an analysis of mouse transcriptome, SREBP-1 was highly expressed in the liver, while its expression in the testis is less than 25%.[37] Moreover, the same relative SREBP-1 expression in these tissues has been reported in rodent and human tissue.[38] The low testicular expression of the transcription factors may explain the lower desaturase response observed in the testis than in the liver.

In conclusion, the results of the present study demonstrate that the conversion of 22:5n-6 to 20:4n-6 was more effective than of 18:2n-6 to 20:4n-6 under the condition of a high 22:6n-3 administration. A second finding in this study is the possibility of 22:5n-6 uptake from the circulation system for testicular 22:5n-6 accumulation during early growth.

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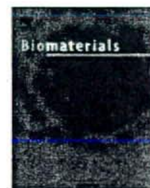

The effect of sulfated hyaluronan on the morphological transformation and activity of cultured human astrocytes

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ABSTRACT

We demonstrated the effect of synthesized sulfated hyaluronan (SHya), which is composed of a sulfated group and hyaluronan, and basic fibroblast growth factor 2 (FGF-2) on normal human astrocytes (NHA) activity and its morphological transformation in vitro study. Astrocyte is a kind of glial cell and stellated astrocyte (activating astrocyte) supports axons network, neurons survival and synaptic plasticity. Treatment of SHya hardly affected NHA proliferation. However combination treatment of SHya and FGF-2 increased NHA proliferation. Treatment of SHya promoted transformation of normal astrocyte into a stella morphology (stellation) and combination treatment of SHya and FGF-2 promoted stellation than that of SHya only. Treatment of SHya increased glial fibrillary acidic protein (GFAP), nestin mRNA and GFAP protein expression in the stellated NHA. The cell–cell adhesion of NHA increased by treatment of SHya. Treatment of SHya increased heparin-binding trophic factors FGF-2, midkine, and some other trophic factors mRNA level in the NHA. These results suggested that the treatment of SHya promoted NHA activity due to enhancing neurotrophins production and the morphological transformation of NHA and the effect of SHya on astrocytes partly involved FGF-2 activity. These findings indicate that SHya may be involved in the astrocyte activity and support neurons survivals.

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1. Introduction

The central nervous system (CNS) comprises neurocytes and glial cells. Recent studies have suggested that glial cells play important roles in neuroprotection and neurogenesis [1–3]. Glial cells not only sustain the neuron network physically but also supply neurotrophic factors to neurons, modify neurotransmission, and support neuroregeneration. Astrocytes, a type of glial cell, intercede between neurons and blood vessels and form the blood–brain barrier. Astrocytes protect axons from injury and promote neuron plasticity by producing trophic factors, such as basic fibroblast growth factor 2 (FGF-2) and glia cell line-derived neurotrophic factor (GDNF) [4,5]. Moreover, astrocytes contribute to neural stem cell (NSC) proliferation and differentiation and the plasticity of neurons. Especially in the region of an injury, normal resting astrocytes transform themselves into activated astrocytes to perform their functions. Activated astrocytes themselves, which have a stellar morphology, increase immunohistochemical reactions of intermediate proteins such as glial fibrillary acidic protein (GFAP) and express the NSC marker nestin, and stellation enhances the production of neurotrophic factors by astrocytes [6,7]. In addition, activation of astrocytes increases the production of FGF-2, midkine, nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), and other trophic factors that contribute to the survival and neurogenesis of neurons and NSC. These results suggest that the activation of astrocytes to supply neurotrophic factors may be an effective therapy for central nervous diseases [4,8] and repair of brain injury.

Glucosaminoglycans have one or more sulfate groups, which have negative charge, and these sulfate groups attract growth factors such as FGF-2 and midkine. Several glucosaminoglycans contribute to the activities of neurotrophic factors. Heparin, a sulfated glucosaminoglycan, binds FGF-2 and enhances FGF-2 bioactivity in the central nervous system [9,10]. A recent study reported that chondroitin sulfate, an oligosaccharide that has sulfate groups, combines with midkine and BDNF to promote neuronal growth in cultured hippocampal neurons [11]. These pleiotrophin-/heparin-binding growth-associated molecules, FGF and midkine, contribute to survival and proliferation of immature and mature central neurocytes and astrocytes. FGF-2 is involved in survival of immature neural progenitor cells, and FGF-2 (20 ng/ml) and epidermal growth factor (EGF) (20 ng/ml) maintained neural stem cell proliferation in vitro [12]. FGF-2 also supported the survival and proliferation of transplanted progenitor cells in the adult rat striatum [13]. It was reported that hippocampal cells are generated over the lifetime of an animal and that FGF-2 stimulates proliferation of these cells to

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a greater extent than EGF [14]. Midkine was identified as a retinoic acid-inducible differentiation factor in an embryonic carcinoma cell line, and with the pleiotrophin-/heparin-binding growth-associated molecules it constitutes a unique family of heparin-binding proteins, which was isolated from rat brains as a neurite outgrowth-promoting protein expressed during the developmental stage of rapid axonal growth [15,16]. These reports suggest that glucosaminoglycans and heparin-binding proteins might be effective in therapy for disorders of the central nervous system and neurogenesis. Thus, we examined the effects of sulfated hyaluronan (SHya), a novel glucosaminoglycan, on astrocyte activity in this study.

Heparin has the largest number of sulfated groups in mammalian tissues, but the number of sulfated groups varies. We synthesized sulfated hyaluronan (SHya) by sulfating hyaluronan, a non-sulfated glucosaminoglycan; these synthesized SHyas have regular numbers of sulfated groups (Fig. 1). In previous studies, we demonstrated the effects of SHya on cell proliferation and differentiation. SHya increased the adhesion molecules *N*-cadherin and connexin43 on the mRNA level in cultured rat calvarial osteoblasts [17]. SHya also enhanced human keratinocyte differentiation and altered mRNA levels of Wnt, Notch1, and Notch3, which play important roles in cell proliferation and differentiation [18]. These studies suggested that SHya could similarly affect glucosaminoglycans that enhance FGF-2 and midkine activities. Therefore, we examined the effects of SHya on normal human astrocytes (NHA) activities, such as proliferation, morphological change, and production of astrocyte trophic factors. In addition, we compared the effects of SHya with that of FGF-2 on astrocyte activities and hypothesized the functional mechanism of SHya.

2. Materials and methods

2.1. Materials

Sulfated hyaluronan (SHya) was synthesized in our laboratory by the method reported previously [18,19]. The molecular weight of SHya was 2.0×10^5, and the degrees of substitution (D.S.) of SHya were 0.4 and 1.0, as determined by the chelate titration method (Fig. 1) [20]. Normal human astrocytes (NHA) were purchased from Cambrex Bio Science Walkersville, Inc. (MD, USA), and NHA culture medium (ABM) (ANG bred kit) was from Sanko Junyaku Co., Ltd., Tokyo, Japan. Human recombinant FGF-2 was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

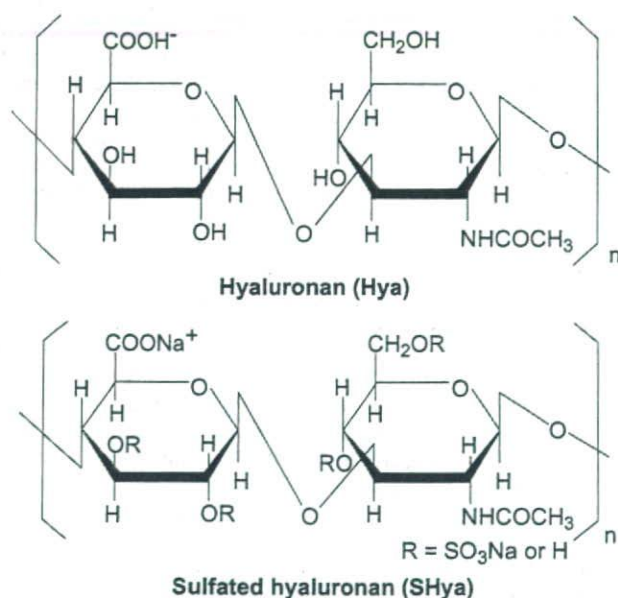

Fig. 1. Structures of hyaluronan (Hya) and sulfated hyaluronan (SHya). SHya is composed of Hya and sulfated groups. The molecular weight of SHya is 2.0×10^5, and the degrees of substitution of SHya were 0.4 and 1.0.

2.2. V79 colony assay

Cytotoxicity was examined using V79 cells by a colony assay following the "Guidelines for Basic Biological Tests of Medical Materials and Devices – Part III: Cytotoxicity tests [21]". Chinese hamster fibroblast V79 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan) and grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a 37 °C humidified atmosphere of 5% CO_2 and 95% air. For the assay, 50 V79 cells/ml were seeded on 24-well plates in Eagle's MEM supplemented with 5% FBS and 1% penicillin–streptomycin. After 24 h incubation in the 37 °C humidified atmosphere of 5% CO_2 and 95% air, 1 ml of the medium with SHya0.4 (0.1, 1, 10, 50, 100 μg/ml), SHya1.0 (0.1, 1, 10, 50, 100 μg/ml) or without (control) was added to each well, and the cells were cultured for 7 days. The colonies formed were fixed with 10% formalin solution and stained with 5% Giemsa solution. The number of colonies on each well was counted, and the efficacy of SHyas was calculated as a ratio of the number of colonies in the sample to that in the control. The data were expressed as an average of four wells, and the procedures were performed in duplicate.

2.3. NHA MTT assay

Effects of SHya on mitochondrial activity of NHA were measured using a microtiter tetrazolium (MTT) assay. NHA cells were extracted from a human fetus at 18 weeks gestation. The basic culture medium was ABM medium supplemented with 5% FBS and recombinant human epidermal growth factor, insulin, GA-1000, ascorbic acid, and L-glutamate (ANG bred kit). NHA were seeded into 24-well plates at a density of 1×10^4/well in ABM medium at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Stock solution of 100 μg/ml SHya1.0 was made directly in ABM medium. After 1 week culture with 0.1, 1, 10, or 100 μg/ml of SHya0.4 or SHya1.0, the medium in each well was replaced with 300 ml of fresh medium containing 6 ml TetraColor ONE reagent (Seikagaku Corporation, Tokyo, Japan). After 2 h, the absorbance at 450 nm/630 nm was measured using a plate reader. The data were expressed as averages of five wells, and the procedures were performed in triplicate.

2.4. Proliferation of NHA cells

NHA were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in ABM (control), ABM supplemented with 10 μg/ml SHya0.4, or ABM supplemented

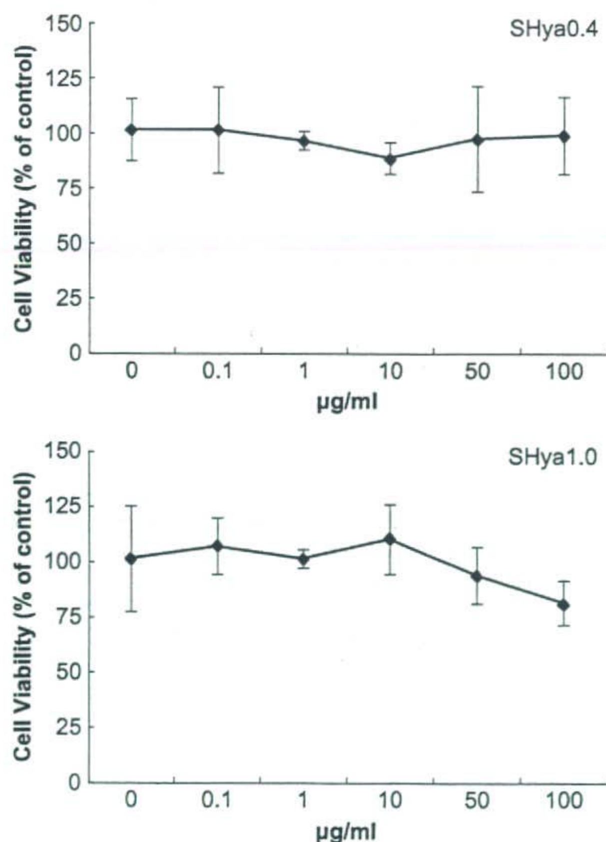

Fig. 2. Viability of V79 cells cultured with SHyas. V79 cells were treated with SHya0.4 or SHya1.0 and cultured for 7 days. Data are expressed as mean ± SD ($n = 4$).

with 10 μg/ml SHya1.0; all three media were supplemented with or without 10 ng/ml FGF-2. Stock solutions of 100 μg/ml SHya and solutions of 1 mg/ml FGF-2 were made directly in ABM medium. The medium was exchanged for a fresh one every 2 days, and NHA were passaged every 7 days. The NHA proliferation data were expressed as averages of three wells.

2.5. Immunocytochemical methods

NHA cells were cultured in ABM (control) or ABM supplemented with 10 μg/ml SHya1.0, and both media were supplemented with or without 10 ng/ml FGF-2 for 10 days. Plated cells were fixed in 4% paraformaldehyde for 30 min and rinsed in phosphate-buffered saline (PBS). Fixed cultures were blocked in 10% blocking reagent (Block Ace Powder; DS Pharma Biomedical Co., Ltd., Osaka, Japan) with 0.3% Triton X-100 and incubated with primary antibodies to GFAP (polyclonal, 1:500, neuron glial cell marker sampler kit; Millipore, Tokyo, Japan) at room temperature overnight. After incubation with the primary antibody, cultures were rinsed in PBS and incubated with fluorescein-conjugated goat anti-rabbit IgG (1:500, Alexa Fluor 488 goat anti-rabbit IgG; Molecular Probes, Eugene, OR, USA) at room temperature for 1 h. After incubation with the second antibody, cultures were rinsed in PBS and incubated for 30 min in 300 nM 4′,6-diamidino-2-phenyl-indole dihydrochloride (DAPI) (Molecular Probes) to stain nucleic acid at room temperature. After incubation, cultures were rinsed in PBS again, and their cells were observed by fluorescence microscopy.

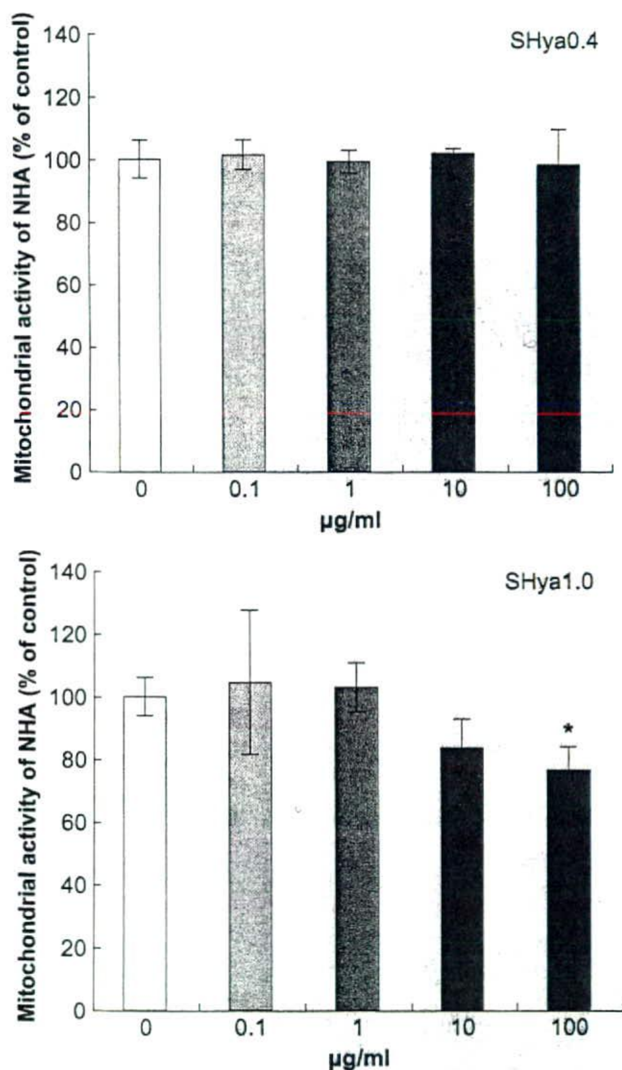

Fig. 3. Mitochondrial activity of NHA cultured with SHyas. Astrocytes were cultured with 0.1, 1, 10, 100 mg/ml SHya0.4 or SHya1.0 for 7 days, and mitochondrial activity was measured by MTT assay. Data are expressed as mean ± SD ($n = 3$). Experimental data showed significant difference from the control group (*, $p < 0.05$).

2.6. Scrape-loading and dye transfer (SLDT) assay

The SLDT technique was performed by the method of El-Fouly et al. [22]. NHA were cultured in ABM for 3 weeks. The medium was changed to a fresh medium containing SHya 48 h before the assay. NHA cells in a confluent monolayer in 35-mm culture dishes were rinsed with Ca^{2+}, Mg^{2+} phosphate-buffered saline [PBS(+)], and the culture dishes were filled with 0.1% Lucifer Yellow (Molecular Probes) in PBS(+) solution and immediately scraped with a sharp scalpel. After 5 min incubation at 37 °C, cells were washed four times with PBS(+), and the extent of the dye influx into cells was monitored using a fluorescence microscope equipped with a type UFX-DXII CCD camera and a super high-pressure mercury

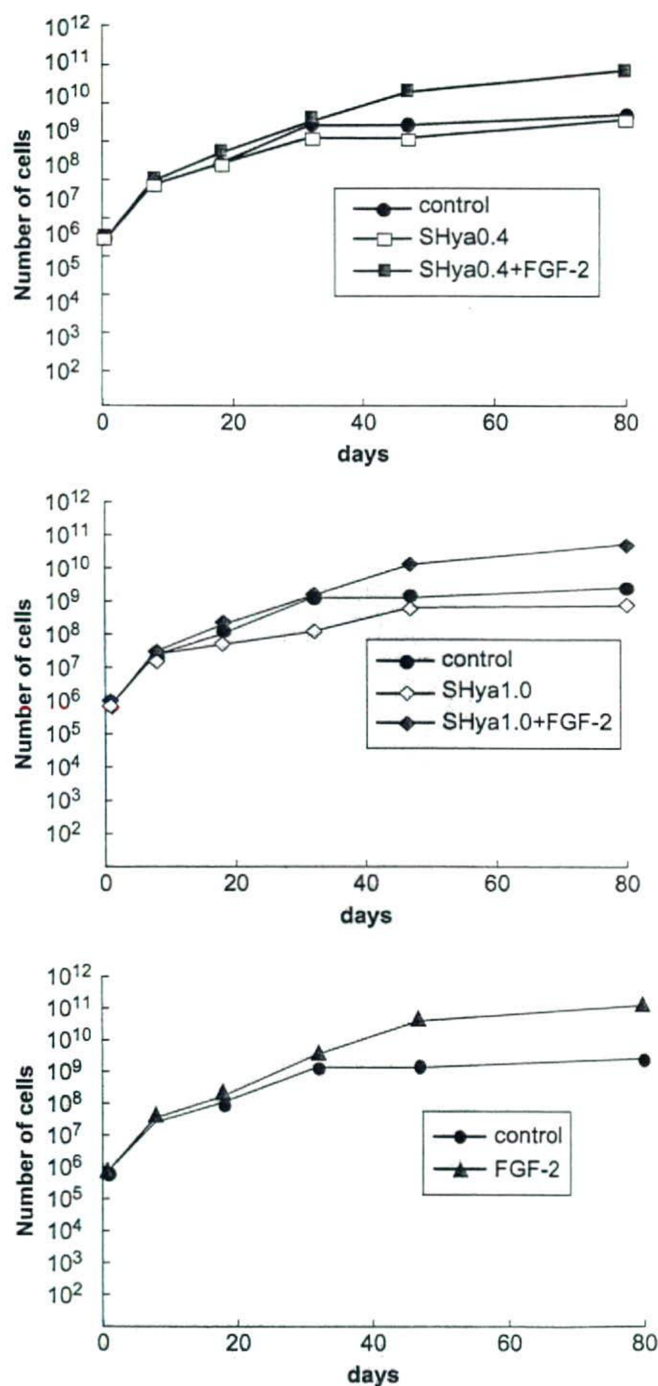

Fig. 4. The effect of SHya, FGF-2, and SHya with FGF-2 on the proliferation of cultured NHA. The concentrations of each regent were 10 μg/ml SHya0.4, 10 μg/ml SHya1.0, and 10 ng/ml FGF-2. Data are expressed as mean ± SD ($n = 3$).

lamp power supply (Nikon Co., Tokyo, Japan). The data were expressed as averages of four wells.

2.7. Expression of neurotrophic factor mRNA

NHA were seeded into six-well plates. When NHA were semi-confluent, the medium was changed to a fresh medium containing SHya. NHA were incubated 24 h at 37 °C, and total RNA was extracted using an RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA). First-strand cDNA was synthesized from total RNA using a first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Midkine and BDNF mRNA were measured quantitatively by a real time PCR light cycler and kit (Roche, Mannheim, Germany). Primer sequences for amplification were 5′-GGCTTGA-CATCATTGGCTGA-3′ and 5′-CCTCCAGCAGAAAGAGAAGAGG-3′ for BDNF, 5′-AGC-CAAGAAAGGGAAGGGAAA-3′ and 5′-TGATTAAAGCTAACGAGCAGACAGA-3′ for midkine. For measurements of FGF-2, NGF, IGF-1 and β-actin mRNA, a human mRNA quantitative 2 step RT-PCR primer set (Search-LC, Heidelberg, Germany) was used. The RNA preparation and real time PCR in the present study were performed in duplicate.

2.8. Statistical analysis

Data for individual groups were expressed as mean ± SD, and two-way ANOVA was performed. The Tukey–Kramer test was used to analyze differences between the control and other groups. In all cases, $p < 0.05$ was considered significant. Results were expressed as mean ± SD.

3. Results

3.1. V79 colony assay

Treatment of SHya0.4 did not alter V79 cell viability. Treatment with 100 µg/ml SHya1.0 tended to decrease V79 cell viability, but the difference was not statistically significant (Fig. 2).

3.2. Mitochondrial activity of NHA

Treatment of SHya0.4 did not alter mitochondrial activity of NHA. Treatment with 100 µg/ml SHya1.0 decreased mitochondrial activity of NHA significantly (Fig. 3).

3.3. Proliferation of NHA cells

NHA cells increased in number for about 30 days (Fig. 4); thereafter, they increased slightly. NHA cell body was enlarged throughout in vitro culture (Figs. 5–7).

Treatment with 10 µg/ml SHya0.4 hardly affected NHA proliferation. Treatment with 10 µg/ml SHya1.0 delayed NHA proliferation, but NHA proliferation had almost caught up that of the control by 50 days. Treatment with 10 ng/ml FGF-2 increased NHA proliferation for 50 days, and cell numbers increased markedly compared with control. In addition, treatment with a combination of SHyas and FGF-2 also increased NHA proliferation without the delay effect of SHya1.0. However, NHA proliferation due to combinations of SHya0.4 or SHya1.0 and FGF-2 was lower than that due to either FGF-2 alone (Fig. 4).

3.4. Morphological change of NHA

Treatment of NHA with 10 µg/ml SHya1.0, 10 µg/ml SHya0.4 with 10 ng/ml FGF-2, or 10 µg/ml SHya1.0 with 10 ng/ml FGF-2 transformed cells into a stella morphology (stellation) in 5 days of culture (Fig. 5). In 10 days of culture, NHA cell bodies were enlarged

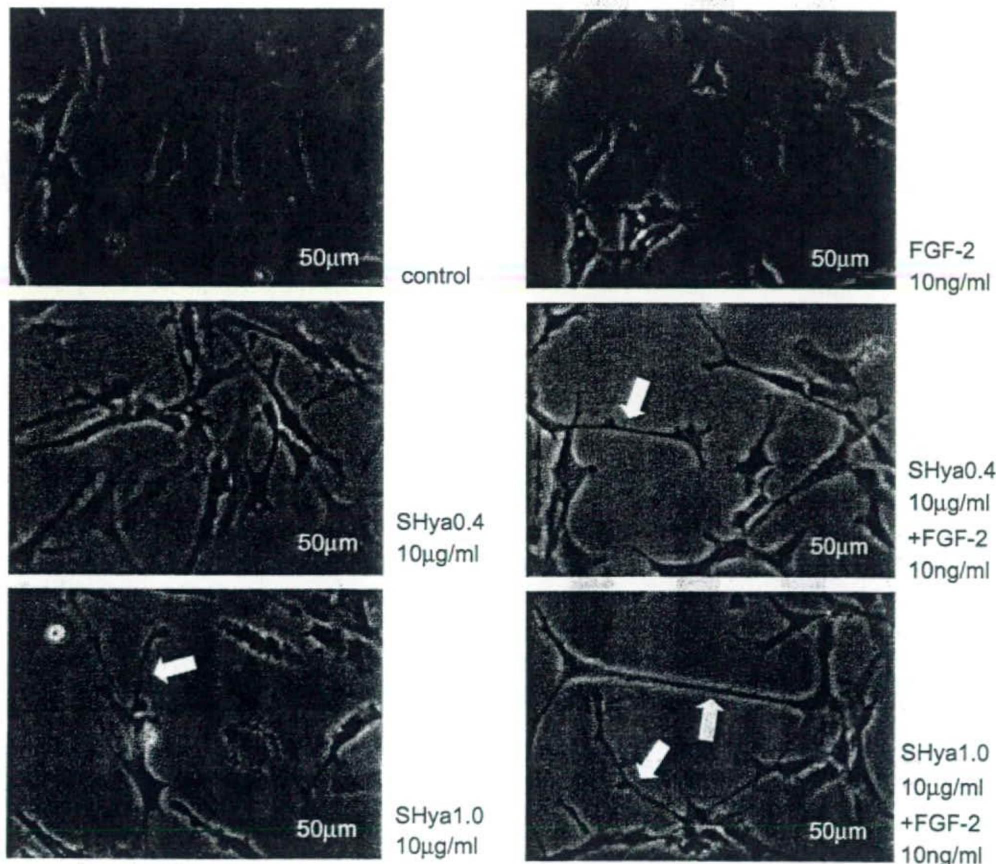

Fig. 5. Effects of SHya, FGF-2, and SHya with FGF-2 on morphological changes in cultured NHA over 5 days. The concentrations of each reagent were 10 µg/ml SHya0.4, 10 µg/ml SHya1.0, and 10 ng/ml FGF-2. The arrow shows stellation of NHA.

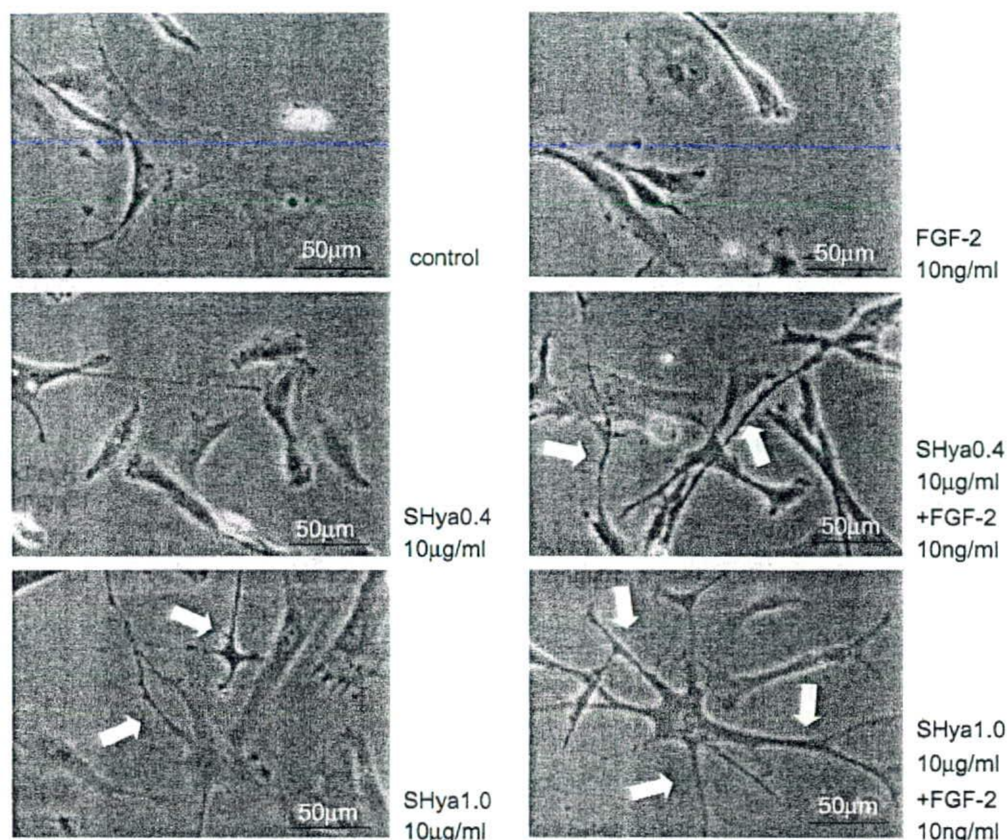

Fig. 6. Effects of SHya, FGF-2, and SHya with FGF-2 on morphological changes in cultured NHA over 10 days. The concentrations of each regent were 10 μg/ml SHya0.4, 10 μg/ml SHya1.0, and 10 ng/ml FGF-2. The arrow shows stellation of NHA.

compared with those at 5 days of culture (control). Treatment with SHya1.0 or SHya0.4 plus FGF-2 promoted NHA stellation, and treatment with SHya1.0 plus FGF-2 promoted NHA stellation dramatically (Fig. 6). After 20 days of culture, NHA cell bodies were enlarged markedly compared with 5 days of culture (control). Treatment with SHya0.4 plus FGF-2 transformed the NHA cell body into a thin cell body like a stella. Treatment with SHya1.0 partly promoted stellation but prevented cell body enlargement. Treatment with SHya1.0 plus FGF-2 promoted stellation markedly. Treatment with FGF-2 alone or FGF-2 with either SHya prevented cell body enlargement (Fig. 7). In 10 days of culture, treatment with SHya, FGF-2, or both increased GFAP and nestin mRNA expression and increased GFAP protein expression. These increases were markedly increased by the combination of either SHya plus FGF-2 (Fig. 8).

3.5. SLDT assay

The SLDT assay estimates cell–cell adhesion and gap-junctional intercellular communication by quantifying dye introduction into cells. Treatment with 10 μg/ml SHya1.0 increased dye influx into NHA significantly (Fig. 9).

3.6. Effect of SHya on mRNA expression

Treatment with SHya1.0 with or without FGF-2 increased FGF-2 and NGF mRNA expression. Treatment with SHya1.0 or FGF-2 increased midkine and IGF-1 mRNA expression. The combination of SHya1.0 and FGF-2 increased midkine mRNA expression remarkably. Treatment with 10 μg/ml SHya1.0 increased BDNF mRNA expression, but the addition of 10 ng/ml FGF-2 prevented BDNF mRNA expression due to SHya1.0 (Fig. 10).

4. Discussion

Current evidence indicates that astrocytes not only help maintain the physical structure of neurons but also contribute to formation of synapses and neural plasticity, in which astrocytes are transformed into active astrocytes (stellation) [5]. Therefore, astrocytes may be an effective target for therapy of central nervous system disorders and synaptic regeneration [23]. The purpose of this study was to elucidate the effects of SHya on astrocytes activities. We synthesized the sulfated hyaluronans SHya0.4 and SHya1.0, which have different numbers of sulfated groups, to estimate the quantitative effects of the number of sulfated groups introduced. First, we examined the biocompatibility and toxicity of SHyas on cultured cells. Nagahata et al. showed that 500 μg/ml synthesized SHya increased alkaline phosphatase activity in rat calvarial osteoblast [17]. A colony assay showed that SHya0.4 and SHya1.0 did not reduce V79 cell viability but treatment with 50 and 100 μg/ml SHya1.0 tended to reduce. MTT assay showed that SHya0.4 did not change mitochondrial activity but treatment with 100 μg/ml SHya1.0 reduced mitochondrial activity of NHA. Treatment with 10 μg/ml SHya1.0 tended to decrease mitochondrial activity, but the difference was not statistically significant. From these results, we suggested that 100 μg/ml SHya declined NHA proliferation and activity and we used 10 μg/ml SHya in the following study. Several glucosaminoglycans contribute to the activities of neurotrophic factors, and heparin, a sulfated glucosaminoglycan, binds FGF-2 and enhances FGF-2 bioactivity in the CNS