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TISSUE SPECIFICITY OF REACTIVITY TO CONCANAVALIN A OF NORMAL HUMAN CELLS IN CULTURE

種々のヒト正常組織から樹立された培養細胞における Concanavalin A に対する反応性の組織特異性

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SUMMARY

An examination was made of the ability of cells that were cultured from various normal human tissues to adsorb concanavalin A (Con A)-treated red blood cells (ConA hemadsorption test). Since most tumor cells have a high reactivity to ConA, the ConA hemadsorption test is often used as a convenient indicator for identifying the neoplastic characteristics in fibroblasts. This study revealed, however, that cultured cells from normal kidney tissue have a remarkably high reactivity to ConA. The morphology of these kidney-derived cells was similar to that of epithelioid cells. However, ConA reactivity of fibroblasts (or fibroblast-like cells) derived from lung, skin, bone marrow, and liver was low. The ConA hemadsorption test is not only useful for identification of neoplastic cells, but also provides information about cell specificity.

INTRODUCTION

Establishment of a simple method for identifying the neoplastic nature of cultured cells is important for the advancement of cancer research. The indexes of neoplastic transformations most frequently used at present include a) loss of postconfluent inhibition of cell proliferation,[1,2] b) proliferative ability in soft agar,[3-7] c) cellular immortality,[8] d) loss of diploidy,[9-11] and e) changes in ConA reactivity.[12] Of these, examination of changes in the reactivity of cells to ConA by using their ability to adsorb ConA-labled red blood cells (C-RBC) has been employed because this procedure

要 約

種々のヒト正常組織から培養した細胞の，concanavalin A (ConA) で処理した赤血球に対する吸着能を調べた (ConA 血球吸着テスト) . 腫瘍細胞の多くが ConA に反応性が高いことから，ConA 血球吸着テストは，線維芽細胞の腫瘍性を同定する上での簡便な一つの指標としてよく用いられている．しかし，本研究では，正常腎臓組織由来の培養細胞が非常に高い ConA 反応性をもつことが分かった．これらの腎臓由来細胞は，上皮様細胞に近い形態をしていた．しかし，肺，皮膚，骨髄及び肝臓由来の線維芽 (様) 細胞の ConA 反応性は非常に低かった．すなわち，ConA 血球吸着テストは，腫瘍性細胞の同定以外に，細胞の特異性を調べる上でも有用な情報を与えるものと思われる．

緒 言

培養細胞の腫瘍性を簡単に同定する方法を確立することは，癌研究の進展上重要である．現在よく使われている腫瘍性形質変換の指標としては，a) 細胞同志の接触による増殖抑制能の消失,[1,2] b) 軟寒天培地中での増殖能の獲得,[3-7] c)無限増殖能の獲得,[8] d) 二倍体性の消失,[9-11] e) ConA 反応性の変化,[12] などが挙げられる．これらのうち，細胞の ConA 反応性の変化を ConA 標識赤血球細胞 (C-RBC) を吸着する能力で調べる方法 (ConA 血球吸着テスト) は，その

(ConA hemadsorption test) is simple and the specificity for neoplastic transformation is high.[1] The method is especially useful to detect tumor-associated characteristics of fibroblasts which can be easily cultured.

Recently, Aizawa et al[13,14] succeeded in employing C-RBC adsorption as an index for the aging of cells in vitro. They found, by examining C-RBC adsorption in cultured fibroblasts derived from normal human fetal lungs, that adsorption was higher in older cells than in younger cells.[13] The number of C-RBC adsorbed by the cells was proportional to the age-related increase in cell membrane surface area.[14] There was no change in the number of ConA receptors per unit area on the membrane surface of the cells used in their study.

Whether C-RBC adsorption of the membrane surface of cultured human cells had tissue specificity was examined. Cells cultured from tissue samples of lung, liver, skin, and bone marrow had a fibroblast morphology (or fibroblast-like cells) and hardly adsorbed any C-RBC. However, most of the cells cultured from the kidney showed an epithelial morphology with strong C-RBC adsorption. Material used in this study was obtained from organs which had been confirmed at autopsy as having neither tumors nor degeneration.

MATERIALS AND METHODS

Tissues and Cells

All cells used in this study were obtained from tissues resected during autopsy and were cultured in our laboratory. Tissue sections were washed thoroughly in a buffer solution containing 500 μg/ml of streptomycin and 500 U/ml of penicillin. These sections were minced into 1 mm^3 or smaller pieces in 5 cm plastic dishes (Falcon Co. USA, Cat. No. 3002) containing a small amount of culture solution. The small pieces were put into Eagle's MEM (Nissui Pharmaceuticals, Tokyo) culture solution containing fetal bovine serum (FBS, Hy-Clone Co., USA), and were cultured at 37°C in 5% CO_2 in air. After 3-10 days, the culture medium was replaced with Eagle's MEM containing 10% FBS. When numerous cells began to proliferate from the pieces of tissue, subcultures were initiated according to methods previously reported.[15,16] Eagle's MEM supplemented with 10% FBS was used for the maintenance and subculture of cells.

手技が簡単なこと，及び腫瘍性形質転換に特異性が高いこと，[1] などから用いられている．特に，培養系で得られやすい線維芽細胞の腫瘍性形質転換を検出できるという意味で有用である．

最近，Aizawa ら[13,14] は，C-RBC吸着能を試験管内細胞老化の一つの指標とすることにも成功した．彼らは，培養された正常ヒト胎児肺由来線維芽細胞のC-RBC吸着能を調べたところ，若い細胞よりも老化した細胞にC-RBC吸着能が高かった．[13] 細胞に吸着したC-RBC数は，加齢に伴う細胞膜表面積の増大に比例していた．[14] すなわち，彼らの用いた細胞の，膜表面の単位面積当たりのConA受容体数には変化がなかった．

我々は，培養されたヒト由来細胞の膜表面上のC-RBC吸着能が組織特異性をもつかどうかを調べた．肺，肝臓，皮膚，骨髄組織より培養した細胞は形態的に線維芽(様)細胞であり，C-RBCをほとんど吸着しなかった．しかし，腎臓から培養したほとんどの細胞は形態的に上皮性を示し，C-RBCを吸着する能力は非常に強かった．本研究で用いた材料は，剖検時に腫瘍あるいは変性をもたないことを確認した臓器から得られた．

材料及び方法

組織と細胞

本研究でのすべての細胞は剖検時に組織を切除し，我々の研究室で培養したものである．組織片は500μg/mlストレプトマイシンと500U/mlのペニシリンを含む緩衝液でよく洗い，少量の培養液を含む5cmプラスチックシャーレ(Falcon社，米国，Cat.No. 3002)の中で1 mm^3 以下の細片に切り刻んだ．細片は胎仔牛血清(Hy-Clone社，米国)を含むEagle MEM(日水製薬，東京)培養液に入れ，5％ CO_2 空気中，37°Cで培養した．3－10日後に，10%胎仔牛血清を含むEagle MEM培地に変え，組織片から多くの細胞が増殖してきたときに，既に報告してある方法[15,16] に従って，継代培養を開始した．細胞の維持と継代培養には，10%胎仔牛血清を添加したEagle MEMを使用した．細胞の剝離には，0.125%

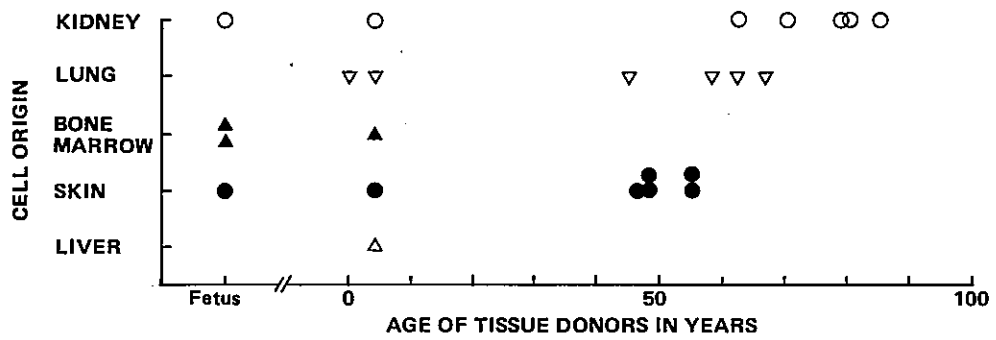

Figure 1. Origin of established cells and age of tissue donors. Each organ was confirmed as having neither tumor nor cellular degeneration at autopsy.

図1　樹立細胞の由来臓器と組織提供者の年齢．各臓器は剖検時に腫瘍と変性のないことを確認した．

A solution containing 0.125% trypsin (Difco, USA) and 0.01% EDTA (Wako Chemicals, Tokyo) was used for cell harvesting. Figure 1 shows the tissues used and the age of the donors.

Preparation of ConA-labeled Red Blood Cells

The method of Oishi et al[12] was employed in the preparation of C-RBC. Two milliliters of male human blood (type A or O) was collected by venipuncture. After suspension in 5 ml of negative phosphate buffer saline, PBS(−), the sample was washed twice by centrifugation for five minutes at 1,000 rpm. Then 0.03 ml of the red blood precipitate was added to 3 ml of PBS in which ConA (Sigma Chemicals, USA, Cat. No. C-2010) had been dissolved at various concentrations. The cells and ConA were allowed to react at 37°C for 30 minutes. After reaction, the red blood cells (RBC) were washed by centrifuging twice as mentioned before, and 3 ml of the PBS(−) was dispensed into each test tube. The C-RBC were freshly prepared for each experiment each day.

ConA Hemadsorption Test

For all cell types, the 5th to the 10th generation of the culture was used in each test. The target cells were cultured in advance for two days in αMEM (GIBCO, USA) culture solution supplemented with 10% FBS, 2.5×10^3 cells were inoculated in wells of the tissue culture chamber with a growing surface area of 1.6 cm^2 (Lab-Tek, USA), and cultured overnight at 37°C in 5% CO_2. After the cells were washed twice in PBS(+), 1 ml of C-RBC was added to each well. After the mixture was allowed to react at 37°C for 10 minutes, the cells were washed three times in PBS(+). If C-RBC remained on

トリプシン (Difco 社，米国) +0.01% EDTA (和光製薬，東京) の溶液を用いた．細胞の由来組織と組織提供者の年齢を図1に示す．

ConA 標識赤血球細胞の作成

基本的には Oishi らの方法[12] に従った．ヒト男性の血液 (A型又はO型) 2ml を静脈穿刺により得た．血液は PBS(−) 緩衝液5ml に懸濁した後，1,000rpm，5分間の遠心操作による洗浄を2回繰り返し，赤血球沈渣の 0.03ml を，種々の濃度の ConA (Sigma Chemicals 社，米国，Cat.No. C-2010) を溶かした PBS 3ml に添加し，37°C で30分間反応させた．反応後，前述と同様に赤血球を2回洗い，各試験管に PBS(−) 3ml を入れた．この C-RBC は，実験ごとに新しく調整し，作成日にだけ使用した．

ConA 血球吸着テスト

すべての種類の細胞について，各テストごとに5−10継代目の細胞を用いた．目的とする細胞はあらかじめ10%胎仔牛血清を添加した αMEM 培養液 (GIBCO 社，米国) で2日間培養しておく．2.5×10^3 個の細胞を組織培養器 (Lab-Tek 社，米国) の穴 (培養面積1.6 cm^2) に播種し，37°C，5% CO_2 条件下で1晩培養した．PBS(+) で細胞を2回洗った後，各穴に 1ml の C-RBC を添加した．37°C で10分間反応させてから，細胞を PBS(+) で3回洗った．培養器表面

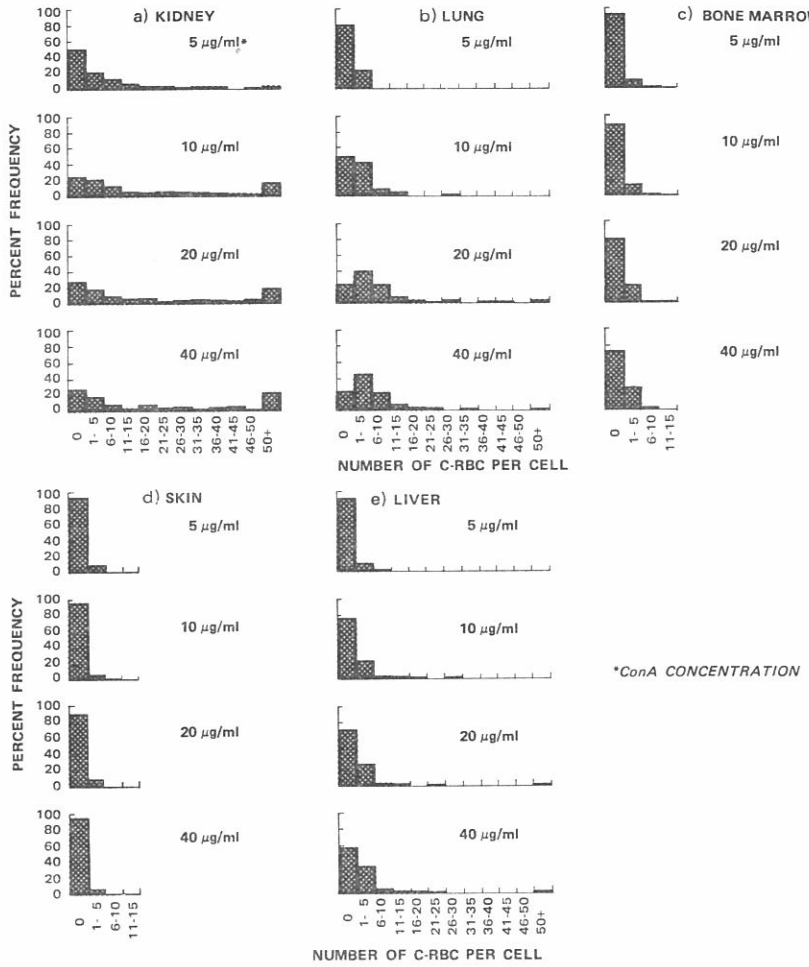

Figure 2. Distribution of C-RBC adsorption of cultured cells derived from organs of a 4-year-old boy, by ConA concentration. a) Kidney, b) Lung, c) Femoral bone marrow, d) Skin, and e) Liver-derived cells.

図2　4歳男子の種々の臓器から樹立した培養細胞のC-RBCに対する吸着分布，ConA濃度別 a）腎臓，b）肺 c）大腿骨骨髄，d）皮膚及びe）肝臓由来細胞．

the surface of the culture chamber, the washing procedure was repeated. Before the cells dried, they were fixed for three minutes in acetone-formalin fixative (pH 6.6). After being washed in water, they were stained with Giemsa's staining solution, and enclosed under a cover glass. The number of C-RBC adsorbed on the surface of the cells was counted using a microscope.

RESULTS

Figure 2 shows the number and frequency of C-RBC adsorbed per cell using various organ-derived cells obtained from the same individual (male aged 4). Cells derived from the bone

上にC-RBCが残っている場合には，洗浄を繰り返した．細胞が乾かない間に，アセトン－ホルマリン固定液（pH 6.6）で3分間固定した．水洗後，ギムザ染色を行い，カバーグラスで封入した．細胞に吸着しているC-RBC数を顕微鏡下で数えた．

結　果

同一個体（4歳，男子）から得られた各種臓器由来細胞を用いての，細胞当たりに吸着するC-RBC数とその頻度を図2に示す．骨髄，皮膚，肝臓由来

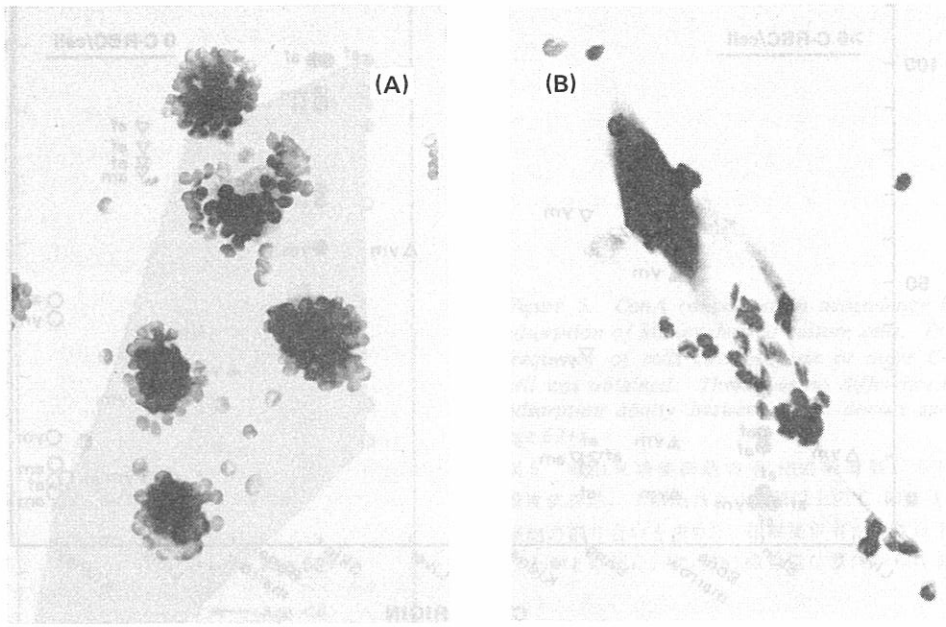

Figure 3. C-RBC adsorption of kidney- and lung-derived cells. After adsorbing C-RBC, the cells were fixed in a pH 6.6 acetone-formalin fixative and stained with Giemsa's solution. A) Kidney-derived cells showed epithelial morphology, and B) Lung-derived cells had a fibroblast-like morphology.

図3　腎由来細胞及び肺由来細胞におけるC-RBC吸着．C-RBC吸着後，細胞はアセトンーホルマリン固定液（pH 6.6）で固定後，ギムザ染色を行った．A)腎由来細胞は上皮性，B)肺由来細胞は線維芽様の形態を示す．

marrow, skin, and liver showed almost no C-RBC adsorption ability, while lung cells showed weak C-RBC adsorption ability. A slight concentration dependency was observed up to a ConA concentration of 20 μg/ml. The above four cell types showed a morphology quite identical with that of fibroblasts. Kidney-derived cells showed remarkably high C-RBC adsorption ability. ConA-unlabeled RBC were not adsorbed by the cells (data not shown). C-RBC at low concentrations (5 μg/ml) were easily adsorbed by kidney cells. However, even when the concentration of ConA was increased to more than 10 μg/ml, adsorption distribution of C-RBC underwent little change. It is evident that cell populations having no C-RBC adsorption exist in a fixed ratio. As a result of the data shown in Figure 2c-e, cells adsorbing six or more C-RBC were regarded as ConA reactivity positive and cells adsorbing no C-RBC as ConA reactivity negative.

Figure 3 shows the C-RBC adsorption on lung and kidney cells. The concentration of ConA in

細胞はほとんどC-RBC吸着能をもたないが，肺由来細胞は弱いC-RBC吸着能を示した．ConA濃度が20μg/mlまではわずかの濃度依存性もみられる．以上の4種の細胞はすべて線維芽細胞の形態を示していた．腎臓由来細胞は非常に大きいC-RBC吸着能を示した．勿論，ConAで標識していない赤血球は全く細胞に吸着されない（データは省略）．低濃度（5μg/ml）のC-RBCは容易に腎由来細胞に吸着された．しかし，ConAの濃度が10μg/ml以上になっても，C-RBCの吸着分布はあまり変わらなかった．すなわち，C-RBC吸着能のない細胞集団がほぼ一定の割り合いで存在することが明らかである．図2c-eに示した結果から，6個以上のC-RBCを吸着する細胞をConA反応性陽性とした．全くC-RBCを吸着しない細胞をConA反応性陰性とする．

肺及び腎臓由来細胞がC-RBCを吸着した様子を図3に示す．C-RBC中のConAの濃度は40μg/mlで

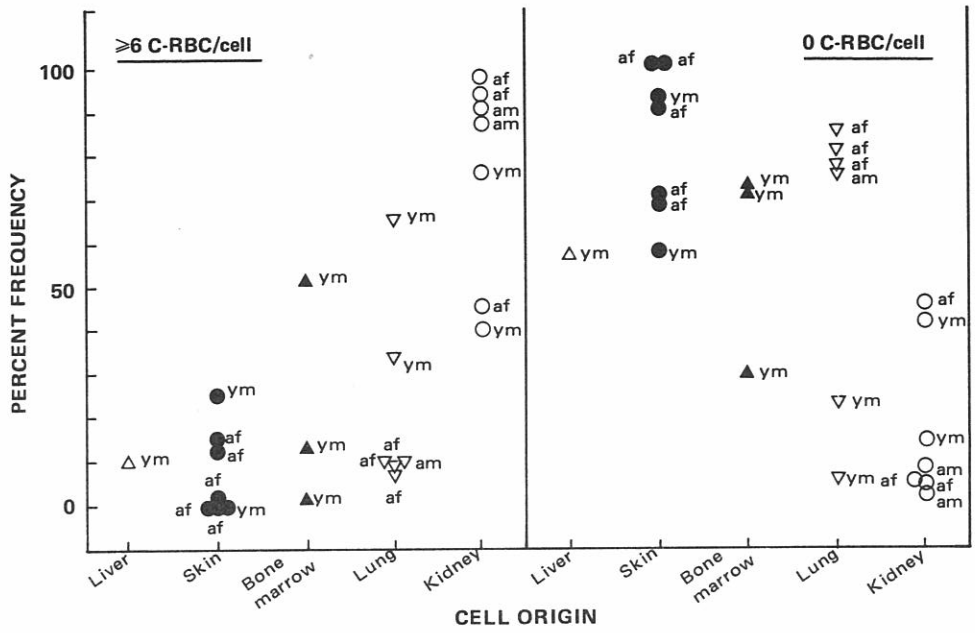

Figure 4. C-RBC adsorption ability of cultured cells established from various normal human tissues. The left panel demonstrates the percent frequency of cells adsorbing six or more C-RBC per cell, and the right panel shows the percent frequency of cells adsorbing no C-RBC.

図4　種々のヒト正常組織から樹立した培養細胞のC-RBCに対する吸着能．左パネルは1細胞当たり6個以上のC-RBCを吸着する細胞の割り合い，右パネルはC-RBCを全く吸着しない細胞の割り合いを示す．

C-RBC was 40 μg/ml. Lung cells were typical fibroblasts, and kidney cells showed epithelial morphology. Vermilion-colored human RBC were adsorbed by cells stained with Giemsa.

The patterns of C-RBC adsorption of cells obtained from various organs of the individuals shown in Figure 1 mostly showed, with the exception of kidney cells, the trends observed in Figure 2b-e. Figure 4 shows in summary the ability to adsorb C-RBC at a concentration of 40 μg/ml. A comparison was made of the percent frequency levels of ConA positive cells (cells adsorbing six or more C-RBC per cell) and ConA negative cells (cells adsorbing no C-RBC). On comparison by age of tissue donors (those less than 4 indicated by y, and those 44 or more indicated by a in Figure 4), there was no association between ConA reactivity and donor age for skin and kidney fibroblasts. Lung fibroblasts showed a tendency for ConA reactivity to be higher in younger cells than in aged cells. However, a larger number of cases are needed in order to detect any cell type

ある．肺由来細胞は典型的な線維芽細胞であり，腎由来細胞は上皮性の形態を示す．ギムザ染色された細胞上に，橙赤色のヒト赤血球が吸着されている．

図1で示された各個体の種々の臓器より得られた細胞のC-RBC吸着能パターンは，腎由来細胞を除いて，大体図2b-eの傾向を示した．40μg/mlのC-RBCに対する吸着能をまとめたのが図4である．ConA反応性陽性（1細胞当たり6個以上のC-RBCを吸着）とConA反応性陰性（全くC-RBCを吸着しない）の細胞の割り合いを比較した．組織提供者の年齢（4歳以下をy，44歳以上をaと図中で表示）を比べると，皮膚線維芽細胞と腎臓線維芽細胞には，ConA反応性と組織提供者年齢との間に関連性はなさそうである．肺線維芽細胞においては，若い細胞の方が，老化細胞よりもConA反応性は高い傾向がみられた．しか

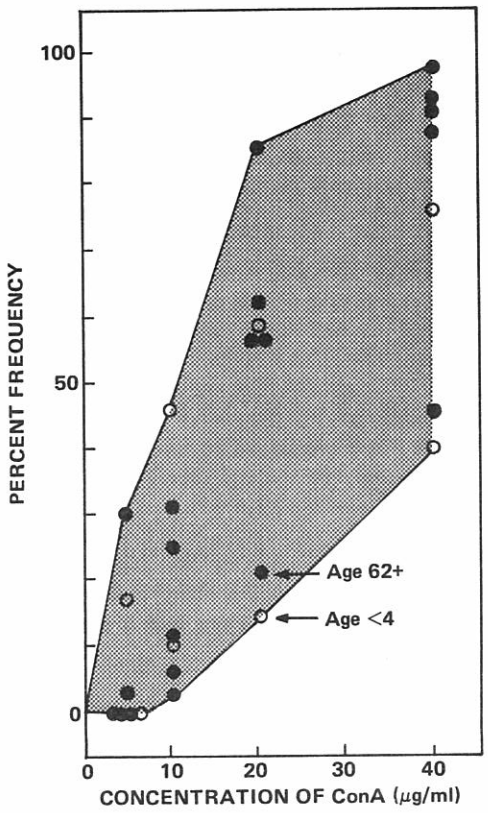

Figure 5. ConA concentration dependency in C-RBC adsorption of kidney-derived culture cells. The percent frequency of cells adsorbing six or more C-RBC per cell was obtained. There was no difference in C-RBC adsorption ability between tissue donors age <4 and age 62+.

図5　腎由来培養細胞のC-RBC吸着能におけるConA濃度依存性．1細胞当たり6個以上のC-RBCを吸着する細胞の割り合いを求めた．組織提供者が4歳以下(○)と62歳以上(●)との間に，C-RBC吸着能に差はみられない．

association between ConA reactivity and donor age. ConA reactivity of skin fibroblasts was low. ConA reactivity of liver and bone marrow cells was also low.

In Figure 5, the percent frequency of kidney cells adsorbing six or more C-RBC per cell is shown in relation to the concentration of ConA. The cells were cultured from a portion of kidney tissue collected from a premature fetus (chromosome karyotype, 44 + XX), a 4-year-old male, a 62-year-old male, a 70-year-old female, a 79-year-old female, an 80-year-old male, and an 85-year-old male (Figure 1). C-RBC adsorption abilities for two cases aged 4 or less, and those for five cases aged 62 or more, were distributed rather widely. That is, there seems to be no correlation between the age of tissue donors and ConA reactivity.

DISCUSSION

Normal human diploid cells, which have a finite doubling potential in culture system, are widely

し，いずれの細胞種においても，ConA反応性と組織提供者の年齢との間に関連性を見いだすためにはもっと多くの例数を必要とする．ともあれ，皮膚線維芽細胞のConA反応性は非常に小さい．肝臓及び骨髄由来細胞もConA反応性は小さい．

一つの腎細胞当たり6個以上のC-RBCを吸着する細胞の割り合いを，ConAの濃度に対応して示したのが図5である．細胞は，未熟児（染色体核型，44＋XX），4歳男子，62歳男性，70歳女性，79歳女性，80歳男性，及び85歳男性の腎臓組織の一部から培養した（図1）．4歳以下の2例と62歳以上の5例におけるC-RBC吸着能は，かなり広い分布にわたって分散している．すなわち，組織提供者の年齢とConA反応性の間に相関関係はなさそうである．

考　察

正常ヒト二倍体細胞は培養系において有限の増殖能をもち，培養系における試験管内の老化モデルと

used as an in vitro model of the aging process. Especially, fibroblasts cultured from lung[15,17,18] and skin tissue from human fetuses[19] and skin tissue from human adults[19] are frequently employed because they are easily available, can be established in culture easily, and have high proliferative ability. Cells derived from other organs are also excellent material for studies of aging.[16] Identification of species specificity or tissue specificity of proliferating fibroblasts and other cells is a meaningful source of information, advancing the study of cellular aging.

Furthermore, to identify the presence or absence of tumor specificity in fibroblasts cultured from tumor tissues is of importance for future cancer studies.

Hayflick and Moorhead[20] and Hayflick[21] suggested the presence of tissue specificity in the in vitro life span of fibroblasts. Oishi et al[12] reported that fibroblasts established from human prostate cancer tissue show high reactivity to ConA.

The present study investigated the ConA reactivity of various cells in order to identify tissue specificity of cells proliferating from normal human tissues. Material was collected only after confirming it to be free of either tumors or degeneration. Cells proliferating from kidney, lung, and skin tissues are easily established, and proliferation of the established cells is good. ConA reactivity of skin fibroblasts is very low, and that of the liver cells and bone marrow cells also seems to be low, and ConA reactivity of lung fibroblasts is slightly higher in younger cells (Figures 2b-e and 4). However, kidney cells showed remarkably high C-RBC adsorption ability compared with the four other types of cells mentioned above. Kidney cells are characterized by their ConA reactivity at low concentrations (5 μg/ml) and the ratio of cells adsorbing 50 or more C-RBC per cell is high. However, many kidney cells do not adsorb C-RBC even when the concentration of ConA used to label human RBC is increased (Figure 2a). Some of the kidney cells that do not adsorb C-RBC demonstrate fibroblast-like morphology, but most are difficult to classify morphologically.

From the above observations, it is evident that ConA reactivity is tissue specific, and especially, that a large number of ConA receptors exist on

して広く使われている．特に，材料の入手が容易なこと，培養樹立の容易さ，増殖能の優れていること，などの理由から，ヒト胎児の肺[15,17,18]及び皮膚組織，[19] ヒト成人の皮膚組織[19]から培養した線維芽細胞がよく用いられている．しかし，その他の臓器由来細胞も優れた老化研究の材料となることは言うまでもない．[16] したがって，増殖してくる線維芽細胞及びその他の細胞の種特異性あるいは組織特異性を同定することは，細胞老化の研究を進める上で重要な意義をもつ．

更に，腫瘍組織から培養した線維芽細胞に腫瘍特異性があるかどうかを同定することも，癌研究を進める上で重要である．

Hayflick と Moorhead[20] 及び Hayflick[21] は，線維芽細胞の試験管内寿命に組織特異性のあることを示唆した．Oishi ら[12] は，ヒト前立腺癌組織より樹立した線維芽細胞では，ConA 反応性が高いことを報告している．

本研究は，ヒト正常組織から増殖してくる細胞の組織特異性を同定する研究の一環として，各種細胞の ConA 反応性を調べた．各材料は，組織に腫瘍や変性のないことを確認して採集された．腎臓，肺，皮膚から増殖してくる細胞の樹立は非常に容易で，樹立細胞の増殖も良好である．皮膚線維芽細胞の ConA 反応性は非常に低く，肝臓及び骨髄細胞の ConA 反応性も低いと思われる．肺線維芽細胞は，若い細胞にやや高い ConA 反応性をもつ（図2b-e 及び4）．しかし，上記4種の細胞に比べて，腎細胞は非常に大きな C-RBC 吸着能をもっていた．腎細胞は低い濃度（5 μg/ml）の ConA で反応し，1細胞当たり50個以上の C-RBC を吸着する細胞の割り合いは非常に大きいことに特徴がある．しかし，赤血球細胞を標識する ConA の濃度を上げても，C-RBC を全く吸着しない細胞も多く含まれている（図2a）．C-RBC を吸着しない腎細胞には，線維芽様の形態を示すものもあるが，多くの細胞については形態を区別するのは困難であった．

以上のことから，ConA 反応性には組織特異性があり，特に腎細胞の膜表面に ConA 受容体が多い

the membrane surface of kidney cells. However, the ConA reactivity of individual kidney cells varied greatly, which suggests that the kidney cells represent a mixed population of various cells with different types of differentiation. However, an overwhelmingly large number of these cells have many ConA receptors on their membrane surface. The number of ConA receptors may change by the cell cycle, but Aizawa et al[14] do not believe this to be true.

Most of the kidney cells had an epithelial morphology which remained stable throughout the in vitro life span of the cells. With some exceptions, it is very difficult to differentiate cultured cells morphologically into epithelial cells and fibroblasts. Particularly, after the first generation of cultured cells has been established and after subculturing is repeated, morphological kinetic changes are frequently observed. The ConA hemadsorption test is useful as an indicator of changes in the growth kinetics of cultured cells.

ことが明らかである．しかし，腎細胞集団中の個々の細胞の ConA 反応性は非常に不均一であった．すなわち，腎臓組織内の分化形態の異なる多種の細胞が混合して増殖してくることを示唆する．しかし，圧倒的に多くの細胞が，多くの ConA 受容体をその膜表面上にもっている．細胞周期によって ConA 受容体数が変化することも考えられるが，Aizawa ら[14] はこの仮説を否定している．

ほとんどの腎臓細胞が上皮様の形態をしており，この形態は試験管内寿命の全体を通して安定していた．一部の例を除いて，培養細胞を形態的に上皮細胞と線維芽細胞とに区別することは非常に困難である．特に，初代培養細胞の樹立後，継代を重ねることによって形態学的な動態変化がしばしば観察される．ConA 血球吸着テストは，培養細胞の動態変化を追求するための一指標としても有用であると思われる．

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