TECHNICAL REPORT
RERF TR 10-84
業 績 報 告 書

IN VITRO CULTURE TECHNIQUES FOR HUMAN THYROID CELLS

ヒト甲状腺細胞の試験管内培養法

RICHARD C. MILLER, Ph.D.
TOSHIO HIRAOKA, M.D. 平岡敬生
HIROMI TENOU, B.S. 天王浩美
KENNETH J. KOPECKY, Ph.D.
MICHAEL P. JONES, M.S.
MICHAEL N. GOULD, Ph.D.

放影研
RERF

RADIATION EFFECTS RESEARCH FOUNDATION
財団法人 放射線影響研究所

A Cooperative Japan - United States Research Organization
日 米 共 同 研 究 機 関

RERF TECHNICAL REPORT SERIES
放影研業績報告書集

The RERF Technical Reports provide the official bilingual statements required to meet the needs of Japanese and American staff members, consultants, and advisory groups. The Technical Report Series is not intended to supplant regular journal publication.

放影研業績報告書は，日米専門職員，顧問，諮問機関の要求に応えるための日英両語による公式報告記録である．業績報告書は通例の誌上発表論文に代わるものではない．

The Radiation Effects Research Foundation (formerly ABCC) was established in April 1975 as a private nonprofit Japanese Foundation, supported equally by the Government of Japan through the Ministry of Health and Welfare, and the Government of the United States through the National Academy of Sciences under contract with the Department of Energy.

放射線影響研究所（元 ABCC）は，昭和50年4月1日に公益法人として発足したもので，その経費は日米両政府の平等分担により，日本は厚生省の補助金，米国はエネルギー省との契約に基づく米国学士院の補助金とをもって運営されている．

放影研 RERF RADIATION EFFECTS RESEARCH FOUNDATION
財団法人 放射線影響研究所

Research Project 研究課題 18-81

IN VITRO CULTURE TECHNIQUES FOR HUMAN THYROID CELLS
ヒト甲状腺細胞の試験管内培養法

RICHARD C. MILLER, Ph.D.[1]; TOSHIO HIRAOKA, M.D. (平岡敏生)[1];
HIROMI TENOU, B.S. (天王浩美)[1]; KENNETH J. KOPECKY, Ph.D.[2];
MICHAEL P. JONES, M.S.[2]; MICHAEL N. GOULD, Ph.D.[3]

RERF Departments of Pathology[1], and Epidemiology and Statistics[2];
University of Wisconsin, Department of Human Oncology[3]
放影研病理部[1]及び疫学統計部[2]; Wisconsin 大学ヒト腫瘍学部門[3]

SUMMARY

Procedures for establishing primary cultures of human thyroid tissue are described. Tissue removed surgically from patients with papillary carcinoma, follicular adenoma, and hyperthyroidism were grown in culture. In addition, normal cells were separated from the margins of the excised tumors and were also cultured. For each gram of thyroid tissue cultured after surgical removal, more than 1×10^5 cells attached to culture dishes. A mixture of 2.5% fetal bovine serum supplemented with insulin, hydrocortisone, transferrin, glycl-l-histidyl-L-lysine acetate, somatostatin, and epidermal growth factor was added to nutrient media containing equal parts of Ham's F-12 and α minimum essential medium. Complete medium selectively supported epithelial cell growth while restricting fibroblast cell growth, especially during the first two weeks of the primary culture. Epithelial and fibroblast cells retained their morphology during the culture period.

Culture conditions that affected the response of cells to X rays were identified. During the culture period, first and second passage cells were compared for differences in their radiosensitivities. In all cases, cells from cancer, adenoma,

要 約

ヒト甲状腺組織の初代培養を確立するための方法について述べた．乳頭状癌，小胞状腺腫及び甲状腺機能亢進症の患者から外科的に切除した組織を培養系で増殖させた．更に，切除した腫瘍の辺縁部から正常細胞を分離し，培養した．外科切除後の培養甲状腺組織1g当たり1×10^5以上の細胞が培養皿に付着した．2.5%牛胎児血清にインシュリン，ヒドロコルチゾン，トランスフェリン，glycl-l-histidyl-L-lysine アセテート，ソマトスタチン及び上皮細胞成長因子を添加した混合液を，Ham F-12とα最少必須培地を同量ずつ含む栄養培地に加えた．この完全培地は特に初代培養の最初の2週間において，上皮細胞の増殖を選択的に支援し，線維芽細胞の増殖を抑制した．培養期間中，上皮細胞と線維芽細胞はその形態を維持した．

X線に対する細胞の反応に影響を与える培養条件を調べた．第一次継代及び第二次継代細胞の放射線感受性の差を比較した．癌，腺腫及び甲状腺機能

Approved 承認 16 May 1984

Printed 印刷 March 1985

normal, and hyperthyroid patients showed differences in their responses to radiation depending on the cell passage number. However, results of replicate experiments of first passage cells that were exposed to X rays showed good agreement between experiments. This technique makes it possible to quantitate the effects of chemical and physical cytotoxic agents on proliferating human thyroid epithelial cells.

INTRODUCTION

Tissue culture methodology has enabled investigators to study the proliferation of cells after exposure to a variety of toxic agents. While, cells from several human tissues and organs have been cultured, most primary cultures were restricted to fibroblast-like cells which could be easily induced to proliferate in culture. As a result, in vitro studies of stromal fibroblasts of various human organs have been numerous in comparison to studies of the response to injury of parenchymal epithelial cells.

The difficulties of growing primary cultures of epithelial cells without fibroblast cell contamination have led to several novel approaches to cell selection. Lasfargues and Moore[1] used periodic applications of collagenase to maintain long-term cultures of mammary cells containing up to 90% epithelial cells. Some investigators used techniques such as substituting citrulline for arginine,[2] D-valine,[3] or a proline analogue[4] to restrict fibroblast cell growth. Others encouraged epithelial cell proliferation using partially defined media consisting of growth hormones and steroids.[5,6] Still others used physical separation techniques including discontinuous density gradients[7,8] and differential filtration techniques to enrich the epithelial cell population in culture.[9] A method to clonally culture human thyroid epithelial cells is reported here. The methodology incorporates several of the techniques mentioned above.

MATERIALS AND METHODS

Cell Origin

Thyroid tissue was obtained from patients undergoing surgery of the thyroid for papillary adenocarcinoma, medullary carcinoma, adenoma, or hyperthyroidism. Normal cells were obtained from those patients with either thyroid cancer or adenoma. Except for those with hyperthyroidism, none of the patients had received therapy prior to surgery. All patients diagnosed

亢進症の患者並びに正常者全例において，細胞の継代に応じて，細胞の放射線反応に差が見られた．しかしながら，第一次継代細胞にX線を照射した反復実験の結果によると，実験間の一致度は高かった．この技法では，増殖中のヒト甲状腺上皮細胞の化学的，物理的細胞傷害薬品の効果を測定することができる．

緒　言

組織培養法によって，種々の傷害薬品に被曝した後の細胞増殖を研究することが可能になった．幾つかのヒト組織及び臓器由来細胞の培養は行われたが，ほとんどの初代培養は，培養系で容易に増殖可能な線維芽様細胞に限定された．その結果，種々のヒト臓器の間質線維芽細胞の試験管内研究は，実質上皮細胞の傷害に対する反応についての研究に比べて膨大な数になっている．

線維芽細胞を混入させることなく上皮細胞の初代培養を行うことは困難なので，特定細胞を選択的に増殖させる新しい試みがなされている．Lasfargues と Moore[1] は，約90％の上皮細胞を含む乳腺細胞の長期培養を維持するため，コラゲナーゼを定期的に使用した．線維芽細胞の増殖を抑制するため，アルギニン，[2] D-バリン，[3] 又はプロリン類似体[4] の代わりにシトルリンを用いる技法等を使用した研究者もある．その他には，成長ホルモンとステロイド類を含む低血清培地を用いて上皮細胞増殖を促進させた例がある．[5,6] また，不連続密度勾配遠心や，[7,8] 培養系の上皮細胞集団を濃縮するため，選択的濾過などの分離法を用いた例もある．[9] 本報では，ヒト甲状腺上皮細胞を培養してクローンを得る方法について述べる．この方法は，上記の幾つかの技法を組み合わせたものである．

材料及び方法

細胞源

甲状腺組織は乳頭状腺癌，髄様癌，腺腫，又は甲状腺機能亢進症のため甲状腺の手術を受けた患者から収集した．正常細胞は甲状腺癌又は腺腫患者から得た．甲状腺機能亢進症例を除いて，手術以前に治療を受けた患者はいない．甲状腺機能亢進症と

as having hyperthyroidism were treated for at least one year prior to surgery with methyl-mercaptoimidazole. At the time of surgery their serum thyroid hormone titers were within the normal range.

診断された患者はすべて，手術の前に少なくとも1年間メチルメルカプトイミダゾールによって治療されていた．手術時の同患者の血清甲状腺ホルモン力価は正常範囲内であった．

Media

The basic nutrient medium consisted of equal parts of Ham's F-12 and α minimum essential medium (αMEM). The growth medium was supplemented with glucose (1.5 g/liter), and penicillin (100 IU/ml), and streptomycin (100 μg/ml). In addition, depending on the treatment conditions, 15%, 10%, 5%, or 2.5% fetal bovine serum (FBS), plus six medium supplements including insulin (10 μg/ml), hydrocortisone (20 ng/ml), transferrin (5 μg/ml), glycl-l-histidyl-L-lysine acetate (10 ng/ml), somatostatin (10 ng/ml), and epidermal growth factor (EGF, 10 ng/ml), were added.

培　地

基本栄養培地は，同量ずつのHam F-12とα最少必須培地（αMEM）から成る．この増殖培地にグルコース（1.5 g/ℓ），ペニシリン（100IU/ml）及びストレプトマイシン（100 μg/ml）を添加した．更に，処置条件に応じて15%，10%，5%又は2.5%の牛胎児血清（FBS）並びにインシュリン（10 μg/ml），ヒドロコルチゾン（20 ng/ml），トランスフェリン（5 μg/ml），glycl-l-histidyl-L-lysine アセテート（10 ng/ml），ソマトスタチン（10 ng/ml）及び上皮成長因子（EGF，10 ng/ml）の六つの培地補完剤を加えた．

Preparation of In Vitro Cultures

Surgically removed tissue was placed in sterile centrifuge tubes containing the basic nutrient medium mentioned above for transportation to the laboratory. Thyroid tissue was minced into pieces of about 1 mm^3 and then incubated at 37°C with gentle stirring for two hours in collagenase (Worthington type) that was diluted to 300 mU/ml in saline. Next, the cell suspension was passed through a coarse filter (53 μm average pore size) in order to remove large cell aggregates. The suspension was centrifuged at 1,200 rpm for 10 minutes and the supernatant was discarded while the packed cells were resuspended in fresh growth medium. We found that plating cells at high density helped to increase the yield of epithelial cells while restricting fibroblast proliferation. Therefore, aliquots of cells at high concentration were added to 60 mm diameter culture Falcon dishes containing 4 ml of medium. The dishes were incubated in a humidified 5% CO_2 atmosphere at 37°C.

試験管内培養系の作成

外科的に切除された組織は，上記の基本栄養培地を含む滅菌遠心管に入れて当研究室に送られた．甲状腺組織は約1 mm^3 の大きさに切り刻み，静かに撹拌しながら37℃で約2時間，生理食塩水で300 mU/mlに希釈したコラゲナーゼ（Worthington 型）で培養した．次に，この細胞懸濁液を粗いフィルター（孔の大きさは平均53 μm）にかけ，大きい細胞塊を除去した．この懸濁液を10分間1,200 rpmで遠心分離し，上清液は捨て，底に残った細胞を新鮮な増殖培地で再懸濁した．細胞を高濃度で播種すると，上皮細胞の収量が増加し，線維芽細胞の増殖が抑制されることが分かったので，高濃度の細胞を，4 mlの培地を含む直径60 mmのFalcon培養皿に加えた．この培養皿を37℃，5% CO_2 のインキュベーターで培養した．

After a period to allow cell attachment (about 18 hours), dishes were carefully washed with phosphate buffer solution (PBS) to remove red blood cells, cell debris, and unattached cells. Fresh medium was added to the dishes and the cells were grown in mass culture for four to six days. Using this procedure the yield of cells after overnight attachment was approximately 1.4×10^5 cells/g of tissue (wet weight).

一定期間（約18時間）細胞を付着させた後，培養皿をリン酸緩衝液（PBS）で十分洗浄し，赤血球，細胞片及び未付着細胞を除去した．新鮮な培地を培養皿に添加し，細胞を4ないし6日間混合培養系で増殖させた．この方法を用いて一晩付着させた後の細胞収量は，組織1 g（湿潤重量）当たり約1.4×10^5細胞であった．

Cells at either first or second passage were used throughout the experiments. First passage cells were prepared by trypsinizing nonconfluent primary cells in mass culture in 0.1% trypsin with 0.01% EDTA for five minutes. Second passage cells were derived from first passage cells grown in mass culture.

実験全体を通じて培養第一次継代細胞と第二次継代細胞の両方を使用した．培養第一次継代細胞は，混合培養系の全面成長していない一次細胞を0.01%のEDTAを含む0.1%のトリプシンで5分間処理して作成した．第二次継代細胞は，混合培養系で増殖させた第一次継代細胞から得た．

Cell Growth Studies

In order to quantitate cell growth kinetics, monodispersed cells in suspension were plated in dishes containing one of the four different types of complete medium at a concentration of 2×10^4 cells/dish. One dish from each medium was sampled daily. All remaining dishes were refed after seven days.

Growth curves were fitted to the data by the least squares method. Since saturation appeared to occur at approximately 1×10^6 cells/dish, only observations with less than 9×10^5 cells/dish were included in the analysis, so as not to bias the estimation of growth rates during the period of exponential growth.

細胞増殖に関する研究

細胞の増殖速度を測定するため，懸濁液に均散した単細胞を，4種類の完全培地のうち一つを含む培養皿で，1枚当たり2×10^4細胞の濃度で播種した．各培地より1皿を毎日検査した．残りの培養皿は7日後に培地を交換した．

増殖曲線を，最小自乗法によってこのデータに適合させた．飽和状態は1皿当たり約1×10^6細胞で生じるようであったので，指数関数的増殖期間の増殖率の推定を偏向させないため，1皿当たり9×10^5細胞以下のレベルで解析した．

Epithelial and Fibroblast Cell Identification

The proportion of epithelial and fibroblast cells in culture was determined by morphological criteria. Spindle-shaped cells were scored as fibroblasts in primary culture and stood out distinctly from the more numerous rounded epithelial-like cells. The relationship between the proportion of epithelial cells, duration of culture, and medium constituents, was analyzed by linear logistic regression analysis.

上皮細胞と線維芽細胞の識別

培養系における上皮細胞と線維芽細胞の比率を形態学的基準によって決定した．初代培養では，紡錘形の細胞は線維芽細胞と判定され，より多くの円形上皮様細胞とは明白に区別された．上皮細胞の比率，培養期間及び培地成分の間の関係を線形ロジスティック回帰分析によって解析した．

Clonal Cytotoxicity Assay

Cell cultures were trypsinized and plated in sets of four dishes with sufficient numbers of cells to yield approximately 40 colonies. A cytotoxicity assay was established by exposing cells to ionizing radiation and then quantitating their ability for clonal growth.

Eighteen hours after plating, attached cells (except for those in control dishes) were x-irradiated at room temperature with a Softex x-ray machine operated at 40 kVp and 5 mA with 0.2 mm aluminum external filtration and a dose rate of 6.38 Gy/min. Immediately prior to irradiation all but 0.5 ml of medium was discarded from each dish to facilitate uniform irradiation of the cells by these low energy photons. After irradiation, the dishes were

コロニー形成法を用いた細胞傷害性検定

培養細胞をトリプシン処理し，シャレー当たり約40個のコロニーを産生するのに十分な細胞を播種した．細胞に電離放射線を照射し，クローン増殖能を定量することにより，細胞傷害性検定を確立した．各線量当たり4枚の培養皿を用いた．

播種後18時間目に，40 kVp，5 mAの条件で0.2mmアルミニウム外部フィルターを装着したSoftex X線装置を用いて，室温，毎分6.38 Gyの線量率で，付着細胞（対照用培養皿を除く）にX線を照射した．X線照射の直前に，このような低エネルギー光子によって細胞が均等に照射されるようにするため，0.5 mlぶん残して培養液をすべて各培養皿から

replenished with fresh medium. The same procedure was followed for control dishes. Seven days later, cells were refed with medium and allowed to grow for the remainder of the 14-day incubation period, after which the attached cells were fixed in formalin and stained with Giemsa. The number of colonies per dish was determined by counting only reproductively viable colonies defined as having more than 50 cells. Plating efficiency (PE), or the fraction of cells that formed colonies was calculated by dividing the number of colonies formed by the number of cells plated. The surviving fraction following a specific radiation dose D, was calculated as

除去した．照射後，培養皿に新鮮な培地を補充した．対照培養皿も同様に処理した．7日後，細胞に新しい培地を添加し14日間培養の残りの期間増殖させた．その後付着細胞をホルマリンで固定し，Giemsaで染色した．50細胞以上と規定されている再生産能力のあるコロニーのみを数えて1培養皿当たりのコロニー数を決定した．コロニー形成率（PE），すなわちコロニーを形成した細胞の分画は，形成コロニー数を播種細胞数で割って計算した．特定の放射線量D後の生存分画は，次のように計算された．

$$S_D = (\text{no. of viable colonies at dose D}) / [(\text{no. of cells plated and exposed to dose D}) \times PE]$$

$$S_D = (\text{線量Dにおける生存可能なコロニーの数}) / [(\text{線量Dの照射を受けた播種された細胞数}) \times PE]$$

The survival data was fitted to the multitarget, single-hit model,

この生存データは，多重標的の単一ヒットモデルに適合させた．

$$S(D) = 1 - (1-e^{-D/D_0})^n ,$$

where S(D) is the probability that a cell will form a colony after exposure to dose D, and the survival parameters are mean lethal dose (D_0) and extrapolation number (n). This model was fit by the method of maximum likelihood. It was assumed that, at exposure dose D, the number of colonies formed follows a Poisson probability distribution with expected value N x PE x S(D), where N is the number of cells exposed. Estimates of the quasi-threshold doses D_q, were calculated from estimates of D_0 and n by the formula $D_q = D_0 \times 1n(n)$. If the estimate of n was less than one, D_q was set to zero. Comparisons of survival curves were based on analysis of variance of survival parameter estimates.

ただしS(D)は，1個の細胞が線量Dの照射後一つのコロニーを形成する確率であり，生存パラメーターは平均致死線量（D_0）及び外挿数（n）である．このモデルは最尤法によって適合された．被曝線量がDのとき，形成コロニー数は，期待値N×PE×S(D)（Nは被曝細胞数）のポアソン確率分布に従うと仮定した．類閾値線量D_qの推定値は，D_0及びnの推定値から公式$D_q = D_0 \times 1n(n)$によって計算された．nの推定値が1以下であれば，D_qはゼロとした．生存曲線の比較は生存パラメーター推定値の分散の解析に基づいて行った．

RESULTS

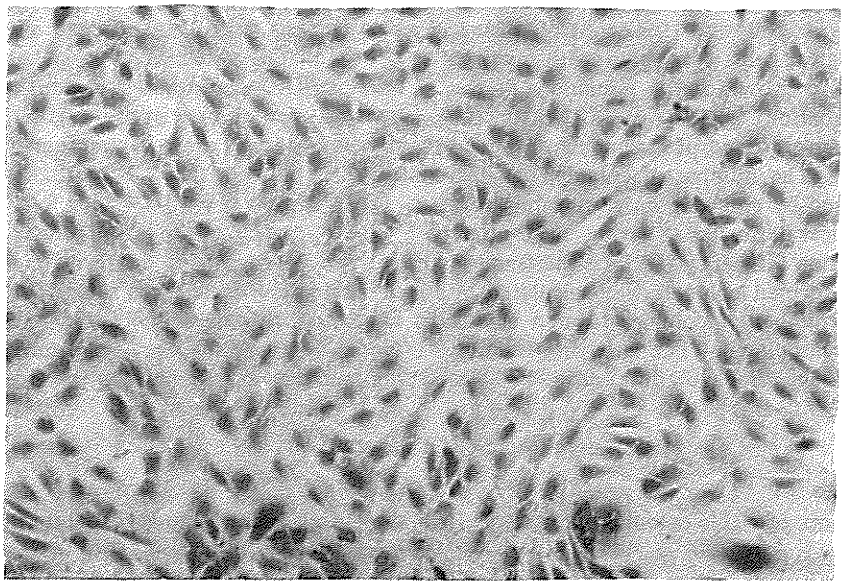
結　果

Cell Morphology

細胞の形態

The typical morphology of human thyroid epithelial cells grown in culture for seven days is shown in Figure 1A. Even after 12 days in culture, epithelial cells retained their characteristic rounded shape, while fibroblast cells

培養系で7日間増殖させたヒト甲状腺上皮細胞の典型的な形態を図1Aに示した．12日間培養した後も，上皮細胞は特徴的な円形を保持し，線維芽細胞は



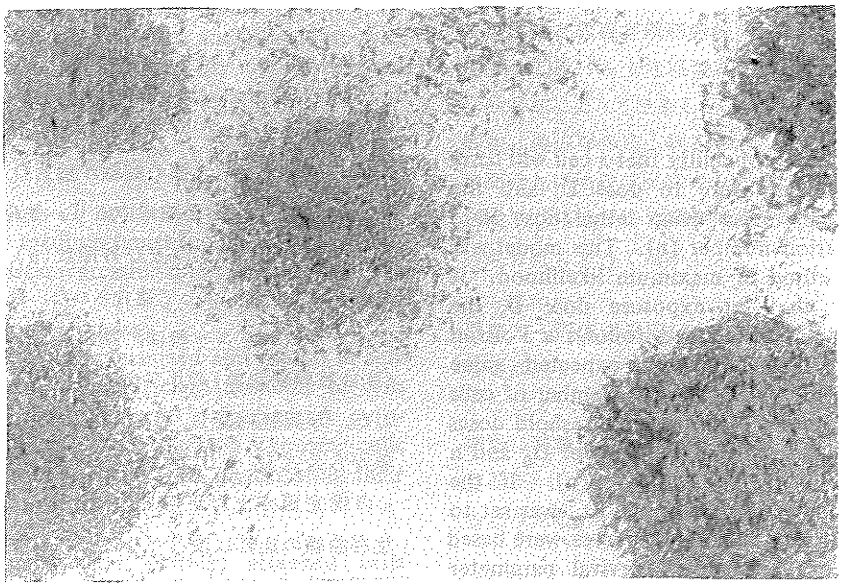


Figure 1. Primary culture of human thyroid cells. A. Cells grown in vitro for seven days; B. Colonies of cells cultured for 14 days.

図1．ヒト甲状腺細胞の初代培養．A．7日間試験管内で増殖させた細胞；B．14日間培養した細胞のコロニー．

remained spindle shaped. Figure 1B shows cell colonies that originated from unirradiated cells after 14 days in culture. Colony sizes varied only slightly in unirradiated cultures; variation was much more marked following high dose irradiation.

Growth Rates and Fibroblast Contamination

Figure 2 shows the population growth in primary culture of one patient's normal thyroid cells grown in a mixture of Ham's F-12 and αMEM, supplemented with 5%, 10%, 15% FBS, or with 2.5% FBS and six growth additives. The growth rate (i.e., the slope of the growth curves) increased significantly with increasing concentration of FBS ($P < 10^{-5}$). Estimated doubling times for cells grown in 15% FBS and 5% FBS were 28 hours and 38 hours, respectively. Cells

紡錘型を維持した．図1Bは，照射されなかった細胞から，培養開始後14日間で発生した細胞コロニーを示したものである．コロニーの大きさは非照射細胞では，わずかな差異しかみられなかったが，高線量照射の場合には大きな差がみられた．

増殖率及び線維芽細胞の混入

Ham F-12とαMEMに5%，10%，15% FBS，又は2.5% FBS及び6種の増殖促進剤を加えた混合液で増殖させた1名の患者の正常甲状腺細胞の初代培養における細胞集団増殖を図2に示した．増殖率（すなわち増殖曲線の勾配）は，FBS濃度の上昇に伴い有意に増加した（$P < 10^{-5}$）．15% FBSと5% FBSで増殖させた細胞の推定倍加時間は，それぞれ28時間と38時間であった．10% FBS，あるいは2.5% FBS，

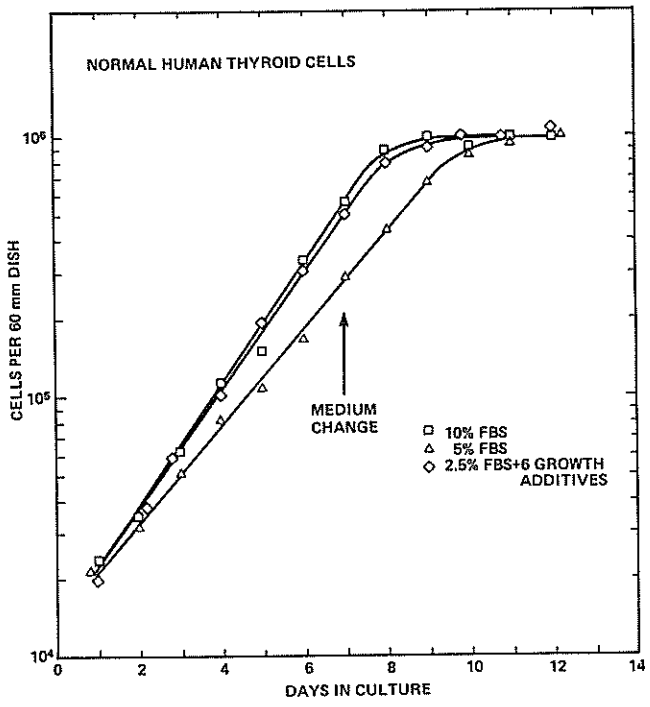


Figure 2. Growth curves of normal human thyroid cells grown in vitro. Growth conditions are described in the text. Medium was replenished seven days after cell plating. Samples were taken daily to determine population doubling.

図2．試験管内で増殖させたヒト正常甲状腺細胞の増殖曲線．増殖条件は本文中に記述した．培地は細胞播種後7日目に交換した．細胞集団の倍加を決定するため毎日細胞数を数えた．

grown in medium supplemented with 10% FBS or with 2.5% FBS plus the six growth additives doubled every 32 hours. Cells in 2.5% FBS with six additives thus grew at a rate significantly greater than that expected in 2.5% FBS without additives ($P < 10^{-5}$); in fact the growth rate in 2.5% FBS with additives was not significantly different from that in 10% FBS without additives (P=.44).

Fibroblast contamination of primary cultures seven days after cell plating was evaluated in cell cultures from 13 patients (Table 1). In all 13 cases, fibroblasts accounted for no more than 12% of the cells. Cancer cell cultures had a somewhat lower average level of fibroblast contamination (4.1%) than normal cells or cells from patients with other diseases (normal 6.4%, adenoma 6.9%, hyperthyroid 6.1%); however this difference is not statistically significant. The evolution of fibroblast contamination over a period of two weeks was observed in six cell strains (Figure 3). Each cell strain was grown both in 10% FBS and in 2.5% FBS with six medium supplements. After accounting for the overall level of fibroblast contamination of each cell strain, the fibroblast contamination was found to be significantly greater, on average, in 10% FBS than in 2.5% FBS with additives ($P < 10^{-6}$). However, this relative advantage of 2.5% FBS with additives was not seen uniformly in all six cell strains; the difference in response varied significantly between cell strains ($P<10^{-6}$).

The results of the radiation cytotoxicity and clonal assay experiments which compared the response to X rays of first and second passage cells from individual patients are summarized in Table 2. In every instance, the PE of cells in second passage was greater than that of the corresponding first passage cells. In all five direct comparisons using normal cells, the cell survival curves of first and second passage cells differed significantly. In the one comparison using adenoma cells, the survival curves were not significantly different; however, the cells involved had an exceptionally low PE. One of two direct comparisons of hyperthyroid cells yielded significantly different cell survival curves. The results in Table 2 suggest no pattern regarding radiosensitivity; in some instances cells in second passage were relatively more radiosensitive, but in others, relatively less radiosensitive, than the corresponding first passage cells.

及び6種の増殖剤を加えた培地で増殖させた細胞は32時間ごとに倍加した．このように6種の増殖剤を加えた2.5% FBSを用いた場合の方が，増殖剤を加えず2.5% FBSを用いた場合よりも，細胞は有意に高率で増殖した（$P<10^{-5}$）．実際，2.5% FBSと増殖剤を併用した場合と，増殖剤を加えずに10% FBSを用いた場合とで増殖率に有意な差はみられなかった（P＝.44）．

細胞播種後7日目の初代培養での線維芽細胞の混入を，13名の患者の細胞培養系について評価した（表1）．13例全員において，線維芽細胞は細胞の12%を占めるにすぎなかった．癌細胞培養系の線維芽細胞混入の平均値（4.1%）は，正常細胞又は他の疾患患者の細胞（正常：6.4%，腺腫：6.9%，甲状腺機能亢進症：6.1%）よりもやや低かった．しかし，この差は統計的に有意ではない．六つの細胞株で，2週間の間に線維芽細胞混入の進行が観察された（図3）．各細胞株は，10% FBSと六つの培地補完剤を加えた2.5% FBSの両方で増殖させた．各細胞株の線維芽細胞混入の総合値についてみると，線維芽細胞混入度は，平均して，増殖剤を加えた2.5% FBSよりも10% FBSの方が有意に高かった（$P<10^{-6}$）．しかしながら，2.5% FBSと増殖剤を併用した場合のこの相対的優位性は，六つの細胞株すべてに均一には認められなかった．反応の差は細胞株によって有意に変動した（$P<10^{-6}$）．

各患者から得た第一次継代細胞及び第二次継代細胞のX線に対する反応を比較した放射線傷害実験，及びクローン検定実験の結果を表2にまとめた．全例において，第二次継代細胞のPEは，対応する第一次継代細胞のそれよりも大であった．正常細胞を用いた五つの直接比較すべてにおいて，第一次及び第二次継代細胞の細胞生存曲線は有意に異なっていた．腺腫細胞を用いた一つの比較において，生存曲線は有意差を示さなかったが，当該細胞は例外的に低いPE値を示した．甲状腺機能亢進症細胞に関する二つの直接比較のうち一つは，有意に異なる細胞生存曲線を示した．表2の結果は，放射線感受性について一定のパターンを示唆するものではない．ある例では，第二次継代細胞は対応する第一次継代細胞より比較的高い放射線感受性を示したが，他の例では，放射線感受性は第二次継代細胞の方が比較的低かった．

TABLE 1 EPITHELIAL AND FIBROBLAST CELLS FROM 7-DAY PRIMARY CULTURES

表1　初代培養7日後の上皮細胞及び線維芽細胞

Patient No.	Cell Type	% Epithelial	% Average
83.02	Normal	91.9	93.6
83.13	"	98.9	
83.18	"	87.5	
83.29	"	96.4	
83.05	Cancer	92.4	95.9
83.18	"	100.0	
83.34	"	96.0	
83.02	Adenoma	97.7	93.1
83.06	"	88.2	
83.11	Hyperthyroid	96.0	93.9
83.14	"	92.3	
83.15	"	98.0	
83.16	"	88.9	

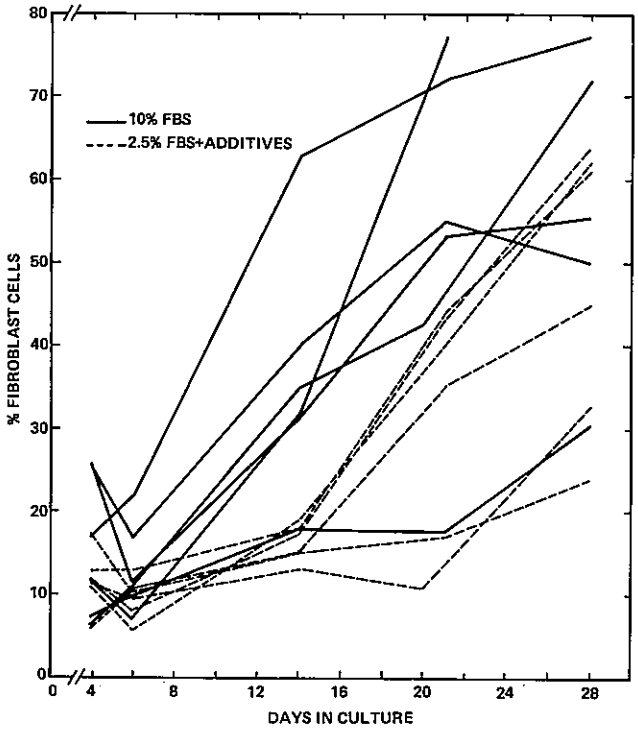


Figure 3. Fibroblast cell growth in human thyroid cultures. Six patient cell cultures were grown in medium supplemented with either 10% FBS or with 2.5% FBS plus six growth additives. Normal, cancer, adenoma, and hyperthyroidism cell cultures were used. Cultures were trypsinized and medium was replaced on days 8, 15, and 22, postplating.

図3．ヒト甲状腺培養系における線維芽細胞の増殖．6人の患者から得た培養細胞を，10% FBS 又は2.5% FBS と6種の増殖剤を添加した培地で増殖させた．正常細胞，癌細胞，腺腫細胞及び甲状腺機能亢進症細胞の培養系を用いた．培養系はトリプシン処理し，培地は播種後8日目，15日目及び22日目に交換した．

TABLE 2 RADIATION RESPONSE TO FIRST AND SECOND PASSAGE THYROID CELLS: COMPARISONS BASED ON INDIVIDUAL PATIENTS' CELLS

表2　第一及び第二次継代甲状腺細胞の放射線反応：各患者の細胞に基づく比較

Patient No.	Passage No.	PE(1)	D_o(3)	D_q(3)	n(3)	χ^2(2)
		NORMAL CELLS				
83.02	1	.180, .190	99.0 ± 1.2	55.4 ± 5.9	1.8 ± 0.1	9.38 **
	2	.232, .246	99.6 ± 1.1	37.0 ± 5.6	1.5 ± 0.1	
83.34	1	.115	77.9 ± 2.4	63.9 ± 10.4	2.3 ± 0.3	22.4 ***
	2	.177	88.2 ± 1.9	60.1 ± 8.1	2.0 ± 0.2	
83.35	1	.099	79.4 ± 2.7	47.2 ± 11.9	1.8 ± 0.3	12.8 *
	2	.114	69.8 ± 2.3	98.4 ± 8.1	4.1 ± 0.6	
83.40	1	.110	79.4 ± 2.6	81.4 ± 9.9	2.8 ± 0.4	21.4 ***
	2	.120	93.4 ± 2.8	61.5 ± 10.7	1.9 ± 0.3	
83.42	1(4)	.074, .078	86.5 ± 2.5	76.3 ± 9.6	2.4 ± 0.3	33.1 ***
	2	.089, .100	77.9 ± 2.0	65.2 ± 7.7	2.3 ± 0.3	
		ADENOMA CELLS				
83.02	1(4)	.001, .003	147.4 ± 10.6	23.1 ± 59.1	1.2 ± 0.5	1.62
	2	.003, .005	130.4 ± 10.5	61.1 ± 53.2	1.6 ± 0.7	
		HYPERTHYROID CELLS				
83.11	1	.037	86.7 ± 4.3	42.1 ± 16.1	1.6 ± 0.3	1.97
	2	.047, .066	82.6 ± 2.3	41.5 ± 8.7	1.7 ± 0.2	
83.41	1	.023	112.5 ± 8.2	21.6 ± 32.4	1.2 ± 0.4	40.2 ***
	2	.079	73.6 ± 3.0	84.2 ± 11.4	3.1 ± 0.6	

(1) Data from simultaneously replicated experiments were combined for estimation of cell survival parameters in this table; in such cases, PE values for both replicates are given.
細胞生存パラメーターの推定のため，同時反復実験は一つにまとめた．その場合は，両反復実験の PE 値を示した．

(2) Based on likelihood ratio test with 2 df. Statistical significance indicated as in Table 3.
自由度 2 の尤度比検定に基づく．統計的有意性は表 3 に示したものと同じ．

(3) Parameter estimate ± SE; D_0 and D_q values given in cGy.
パラメーター推定値 ± SE; D_0 と D_q の値は cGy で示した．

(4) Based on combined data from two replicate experiments on successive days.
連続する 2 日間に行った 2 回の反復実験を一つにまとめたデータに基づく．

Reproducibility of the Assay

The variability of PE as a function of individuals and cell passage number is illustrated in Table 3. Replicate experiments using cells from 12 different patients were conducted to evaluate the reproducibility of PE and of the radiosensitivity assay. The left side of Table 3 gives the results for 12 pairs of experiments (cells from nine patients) in which the two replications were performed simultaneously, while the right side of the table lists the results for six pairs of experiments (cells from five patients) in which the two replications were separated by intervals of from 1 to 18 days. For 2 of the 12 simultaneously replicated experiments, there was a statistically significant difference between the

検定の再現性

症例及び細胞継代数の関数としての PE の変動性を表 3 に示した．PE 及び放射線感受性検定の再現性を評価するため，12人の異なる患者から得た細胞を用いて反復実験を行った．表 3 の左側は，2 回の反復実験を同時に行った12組の実験（9 人の患者から得た細胞）の結果を示し，右側は，2 回の反復実験を 1 日から18日間の間隔を置いて実施した 6 組の実験（5 人の患者から得た細胞）を示したものである．12の同時反復実験のうち二つの実験で，PE 値に

two PE values. In both instances the cells originated from hyperthyroid patients; in one case the cells were in second passage, and in the other case the cells were late in first passage. Four of the six pairs of experiments replicated on different days had significant variation in PE. In both of the other pairs, the second replicate experiment was begun on the day after the first experiment.

The estimates of cell survival parameters for the 12 pairs of simultaneously replicated experiments

統計的に有意な差がみられた．両例とも，細胞は甲状腺機能亢進症由来であった．このうち一方は第二次継代細胞で，他方は第一次継代培養後期の細胞であった．異なる日に反復して行った6組の実験のうち，4組はPEに有意な変動を示した．他の2組の場合，2回目の実験は1回目の翌日に行われた．

12組の同時反復実験に対する細胞生存パラメーターの

TABLE 3 REPRODUCIBILITY OF PLATING EFFICIENCY(1)
表3　コロニー形成率の再現性(1)

Simultaneous Replications			Replications on Different Days			
Patient No.	Passage No.	Plating Efficiency	Patient No.	Passage No.	Interval(2)	Plating Efficiency
			NORMAL CELLS			
83.02	1	0.180, 0.190	83.35	1	8	0.058, 0.099***
83.13	1	0.122, 0.125	83.42	1	1	0.078, 0.074
83.02	2	0.232, 0.246	83.34	2	6	0.177, 0.207*
83.42	2	0.089, 0.100	83.40	2	18	0.120, 0.073***
			CANCER CELLS			
83.05	2	0.141, 0.156	–	–	–	–
			ADENOMA CELLS			
83.02	2	0.005, 0.003	83.02	1	1	0.003, 0.001
			83.42	1	2	0.139, 0.090***
			HYPERTHYROID CELLS			
83.14	1	0.092, 0.098	–	–	–	–
83.15	1	0.080, 0.090	–	–	–	–
83.16	1	0.062, 0.071	–	–	–	–
83.45(3)	1A	0.053, 0.045	–	–	–	–
	1B	0.086, 0.128***	–	–	–	–
83.11	2	0.047, 0.066*	–	–	–	–

(1) Statistical significance of differences between PE for each of two replicate experiments is given by
2回の反復実験それぞれに対するPEの統計的有意差は，次のように示される：
Sug .01<P<.10, * .001≤P<.01, ** .0001≤P<.001, *** P<.0001

(2) Number of days between replicate experiments.
反復実験の間の日数

(3) The 1B replications were performed seven days after the 1A replications.
1B反復実験は1A反復実験の7日後に行われた．

are given in Table 4. For one replicate pair, there was a significant difference between the results of two assays performed simultaneously; for two other replicate pairs, the differences were of marginal significance. In all three of these cases, the assays were based on second passage cells; however the PE values within each of the three pairs did not differ significantly (Table 3).

推定値を表4に示した．1組の反復実験では，同時に行われた二つの検定の結果に有意な差が認められたが，他の2組では，その差にはほとんど有意性がなかった．これら3例すべてにおいて，検定は第二次継代細胞に対して行われたが，3組それぞれの間にPE値の有意な差は認められなかった（表3）．

TABLE 4 REPRODUCIBILITY OF SIMULTANEOUSLY REPLICATED CELL SURVIVAL ASSAYS

表4　同時に繰り返された細胞生存検定の再現性

Patient No.	Passage No.	D_0(2)	D_q(2)	n(2)	χ^2(1)
		NORMAL CELLS			
83.02	1	101.3 ± 1.7	44.7 ± 8.2	1.6 ± 0.1	4.54
		96.4 ± 1.7	67.8 ± 8.4	2.0 ± 0.1	
83.13	1	82.2 ± 2.2	76.8 ± 8.9	2.5 ± 0.3	0.49
		80.9 ± 2.2	76.1 ± 9.2	2.6 ± 0.3	
83.02	2	95.6 ± 1.6	48.4 ± 7.4	1.7 ± 0.1	13.01 *
		103.7 ± 1.7	24.8 ± 8.4	1.3 ± 0.1	
83.42	2	88.6 ± 3.6	64.4 ± 13.9	2.1 ± 0.4	0.21
		84.3 ± 3.5	88.0 ± 13.0	2.8 ± 0.5	
		CANCER CELLS			
83.05	2	89.3 ± 2.6	39.7 ± 10.5	1.6 ± 0.2	5.56 Sug
		81.1 ± 2.4	62.0 ± 9.4	2.1 ± 0.3	
		ADENOMA CELLS			
83.02	2	115.4 ± 11.0	95.3 ± 59.0	2.3 ± 1.3	6.62 Sug
		170.2 ± 25.9	–	0.8 ± 0.5	
		HYPERTHYROID CELLS			
83.14	1	73.5 ± 2.8	102.6 ± 11.0	4.0 ± 0.8	3.80
		66.2 ± 2.7	115.0 ± 9.6	5.7 ± 1.1	
83.15	1	124.4 ± 5.1	7.4 ± 19.8	1.1 ± 0.2	1.45
		122.5 ± 4.9	–	1.0 ± 0.2	
83.16	1	94.2 ± 3.6	99.6 ± 15.5	2.9 ± 0.6	0.29
		92.1 ± 3.5	111.0 ± 14.5	3.3 ± 0.7	
83.45(3)	1A	83.4 ± 4.3	–	0.9 ± 0.2	0.28
		83.5 ± 4.5	3.5 ± 17.0	1.0 ± 0.2	
	1B	97.2 ± 3.4	21.4 ± 13.2	1.2 ± 0.2	0.31
		99.7 ± 3.1	13.3 ± 12.2	1.1 ± 0.1	
83.11	2	82.3 ± 3.0	44.5 ± 10.5	1.7 ± 0.2	0.37
		83.4 ± 3.7	34.0 ± 15.7	1.5 ± 0.3	

(1) Based on likelihood ratio test with 2 df. Statistical significance indicated as in Table 3.
自由度2の尤度比に基づく．統計的有意性は表3に示したものと同じ．

(2) Parameter estimates ± SE; D_0 and D_q values given in cGy.
パラメーター推定値± SE; D_0 と D_q の値は cGy で示した．

(3) The 1B replications were performed seven days after the 1A replications.
1B反復実験は1A反復実験の7日後に行われた．

The results for the six pairs of nonsimultaneous replications are given in Table 5. In these cases, irreproducibility of the cell survival assay corresponded to that seen for the PE; in particular, when two replicate experiments were begun on successive days, the resulting survival curves were not significantly different.

6組の非同時性反復実験の結果を表5に示した．これらの実験において，細胞生存検定の非再現性はPEの場合と対応した．特に，二つの反復実験が連続した両日に実施されると，その結果得られる生存曲線は有意差を示さなかった．

TABLE 5 REPRODUCIBILITY OF CELL SURVIVAL ASSAYS REPLICATED ON DIFFERENT DAYS

表5　異なる日に反復して行われた細胞生存検定の再現性

Patient No.	Passage No.	Interval(1)	D_0(3)	D_q(3)	n(3)	χ^2(2)
			NORMAL CELLS			
83.35	1	8	79.6 ± 3.3	108.9 ± 13.4	3.9 ± 0.8	29.4 ***
			79.4 ± 2.7	47.2 ± 11.9	1.8 ± 0.3	
83.42	1	1	88.6 ± 3.6	64.4 ± 13.9	2.1 ± 0.4	1.58
			84.3 ± 3.5	88.0 ± 13.0	2.8 ± 0.5	
83.34	2	6	88.2 ± 1.9	60.1 ± 8.1	2.0 ± 0.2	21.5 ***
			86.3 ± 1.8	32.3 ± 7.6	1.5 ± 0.1	
83.40	2	18	93.4 ± 2.8	61.5 ± 10.7	1.9 ± 0.3	5.74 Sug
			89.6 ± 3.9	49.8 ± 16.0	1.7 ± 0.3	
			ADENOMA CELLS			
83.02	1	1	154.7 ± 16.5	−	1.0 ± 0.6	0.39
			141.5 ± 13.5	43.4 ± 72.2	1.4 ± 1.7	
83.42	2	2	88.0 ± 2.6	60.3 ± 10.3	2.0 ± 0.3	23.0 ***
			101.5 ± 3.8	56.3 ± 15.0	1.7 ± 0.3	

(1) Number of days between replicate experiments.
反復実験の間の日数

(2) Based on likelihood ratio test with 2 df. Statistical significance indicated as in Table 3.
自由度2の尤度比に基づく．統計的有意性は表3に示したものと同じ．

(3) Parameter estimate ± SE; D_0 and D_q values given in cGy.
パラメーター推定値±SE; D_0とD_qの値はcGyで示した．

DISCUSSION

Methods have been established for culturing primary human thyroid epithelial cells in both mass and clonal culture. A mixture of medium and 2.5% FBS supplemented with growth additives provides cells with the nutrients and hormones necessary to encourage epithelial cell growth. Epithelial cells retain their morphology in culture and are the dominant cell type for at least three weeks. Their distinctive shape during colony formation make them easy to identify (Figure 1).

考　察

ヒト甲状腺上皮初代培養細胞の混合培養法並びにクローン培養法を確立した．培地と増殖剤を添加した2.5% FBSの混合液により，上皮細胞の増殖を促進するのに必要な栄養及びホルモンが細胞に供給される．上皮細胞は培養系でもその形態を保持し，少なくとも3週間は主要な細胞型である．この細胞は，コロニー形成中明白な形状を示すので，容易に識別できる（図1）．

As shown in Figure 3, fibroblast cell growth over a two-week period can be reduced relative to epithelial cell growth when the defined growth medium is used. This is especially true if, during the establishment of the primary cultures, cells are plated at high density. As cells are sub-cultured repeatedly, fibroblast cells proliferate and eventually become the predominant cell type. However, if experiments are performed using cell cultures that are no more than two weeks old, greater than 90% of the resulting colonies will be epithelial.

The PE values for patients selected to represent all thyroid disease groups varied widely. Within each group, however, PE values were less variable. Also, when cells were sampled repeatedly at the same passage and on the same day, PE values were similar.

The length of time and manipulation of cells in culture can have a profound effect on cell radiosensitivity. Although the degree and direction of change between first and second passage cell radiosensitivities is not consistent, it is clear that for all thyroid cell conditions examined, significant differences occurred.

Within the experimental conditions discussed, it is now possible to mass-culture human thyroid epithelial cells for a prolonged period of time. In addition, we have developed methods for reproducible clonal culture of these cells. Consistent results can be obtained that will allow comparative cytotoxicity studies of normal and neoplastic human thyroid epithelial cells.

図3に示すように，規定の培地を用いると，2週間の線維芽細胞増殖は，上皮細胞増殖に比例して減少させることができる．これは，初代培養系の確立時に細胞が高濃度で播種されると，特にいえることである．細胞は繰り返し継代培養されるにつれ，線維芽細胞が増殖し，最終的には主要な細胞型になる．しかし，2週間以内の細胞培養系を用いて実験を行うと，その結果得られるコロニーの90％以上は上皮細胞で占められるであろう．

甲状腺疾患群全体を代表するように選択された患者のPE値には，相互に大きな差があった．しかしながら，各群内におけるPE値の変動は少なかった．また，同じ継代で同じ日に細胞を繰り返し実験に供すると，PE値は類似したものになった．

細胞培養の時間と方法は細胞の放射線感受性に大きな影響を及ぼし得る．第一次及び第二次継代細胞の間の放射線感受性の変化の程度と方向は一定ではないが，検討した甲状腺細胞の状態すべてにおいて有意な差が生じたことは明白である．

ここに述べた実験条件の範囲内において，ヒト甲状腺上皮細胞を長期間混合培養することが可能である．更に，この細胞の再現性のあるクローン培養法も開発した．したがって，正常及び腫瘍性のヒト甲状腺上皮細胞の細胞傷害性に関する比較研究を可能にする一貫した結果が得られる．

REFERENCES
参考文献

1. LASFARGUES EY, MOORE DH: A method for the continuous cultivation of mammary epithelium. In Vitro 7:21-5, 1971

2. SUN NC, SUN CRY, TENNANT RW, HSIE AW: Selective growth of some rodent epithelial cells in a medium containing citrulline. Proc Natl Acad Sci USA 76:1819-23, 1979

3. GILBERT SF, MIGEON BR: D-valine as a selective agent for normal human and rodent epithelial cells in culture. Cell 5:11-17, 1975

4. KAO W, PROCKOP DJ: Proline analogue removes fibroblasts from cultured mixed cell populations. Nature 266:63-4, 1977

5. AMBESI-IMPIOMBATO FS, COON HG: Thyroid cells in culture. Int Rev Cytol (Suppl) 10:163-72, 1979

6. AMBESI-IMPIOMBATO FS, PARKS LAM, COON HG: Culture of hormone-dependent functional epithelial cells from rat thyroids. Proc Natl Acad Sci USA 77:3455-59, 1980

7. CLYNES MM, SMYTH H, O'KENNEDY R, CORRIGAN A: Separation of viable from nonviable cells using a discontinuous density gradient. In Vitro 16(9):809-12, 1980

8. SKYES JA, WHITESCARVER J, BRIGGS L, ANSON JH: Separation of tumor cells from fibroblasts with use of discontinuous density gradients. J Natl Cancer Inst 44:855-64, 1970

9. NITSCH L, WOLLMAN SH: Suspension culture of separated follicles consisting of differentiated thyroid epithelial cells. Proc Natl Acad Sci USA 77:472-6, 1980