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SENSITIVITY TO MITOMYCIN-C AND RADIATION OF CELLS DERIVED FROM PATIENTS WITH FAMILIAL COLON POLYPOSIS: AN AUTOSOMAL DOMINANT HEREDITARY DISEASE

常染色体性優性遺伝疾患である家族性大腸ポリポーシス患者から得られた細胞のマイトマイシン-C及び放射線に対する感受性

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SUMMARY

This study was undertaken to investigate the sensitivity to mitomycin-C (MMC) of skin fibroblasts derived from patients with adenomatosis coli (AC), especially familial colon polyposis. The sensitivity to X rays and ultraviolet rays of AC cells cultured at RERF was similar to that of normal human diploid cells. However, there were large individual differences in sensitivity to MMC. DNA elongation in cells sensitive to MMC was found to be inhibited after MMC treatment. Sites highly sensitive to MMC were considered to be involved in the initial stages of DNA synthesis.

INTRODUCTION

Organisms have mechanisms to repair DNA damage which thereby act to reduce hereditary toxicity. Repair mechanisms are genetically controlled and many mutants deficient in such DNA repair have been detected. A representative case is xeroderma pigmentosum.[1] Cocayne's syndrome,[2,3] ataxia telangiectasia,[4] Fanconi's anemia,[5] and Bloom's syndrome[6] are also strongly suspected of being cancer-prone hereditary diseases associated with DNA repair deficiency. Each of these hereditary diseases shows a pattern of autosomal recessive hereditary.

要 約

本研究は大腸腺腫症，特に家族性大腸ポリポーシスと診断された患者から得られた皮膚線維芽細胞を用いて，マイトマイシン-C(MMC)に対する感受性を調べた．当所で培養樹立した大腸腺腫症細胞のX線及び紫外線に対する感受性は，ヒト正常二倍体細胞のそれと同じであった．しかし，MMCに対する感受性には大きな個人差があった．MMC高感受性細胞においては，MMC処理後のDNA鎖の伸長が著しく抑制されることが分かった．すなわち，DNA合成開始期にMMC高感受性部位が存在することが考えられる．

緒 言

生物はDNA損傷に対する多数の遺伝修復機構を備えており，DNA損傷による遺伝毒性を減ずるように働いている．これらの修復機構は遺伝的にコントロールされているために，DNA修復機構の欠損した突然変異体も多く見付かっている．代表例には色素性乾皮症がある．[1] Cocayne 症候群，[2,3] 失調性毛細管拡張症，[4] Fanconi 貧血，[5] Bloom症候群[6] なども，DNA修復欠損に関連した高発癌性遺伝病である疑いが強く，いずれも常染色体性劣性遺伝の様式を示す．

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While much is known about the autosomal recessive hereditary diseases associated with DNA repair deficiency, little is known about the mechanism involved in the increased carcinogenic potential associated with dominant hereditary diseases. Weichselbaum et al[7,8] reported that skin fibroblast derived from patients with D-deletion type (13q−) retinoblastoma is highly sensitive to X rays. This report is important because it shows that genes associated with DNA damage repair apparently are located on the deleted region of the long arm of chromosome 13. However, insofar as results contradicting this report have also been reported,[9,10] further follow-up studies are deemed necessary.

AC is a disease in which diffuse adenomas develop extensively throughout the large intestine. It is classified into familial colon polyposis, Gardner's syndrome, and Turcot's syndrome, according to the presence or absence of concomitant lesions.[11] Familial colon polyposis and Gardner's syndrome,[12] both of which are autosomal dominant hereditary diseases, are characterized by development of cancer of the large intestine with high frequency and also by being accompanied by other concomitant neoplastic lesions.[11] Turcot's syndrome, a rare disease, is an autosomal recessively inherited trait.[13] Little et al[14] and others[15,16] have reported that skin fibroblasts derived from patients with familial colon polyposis and Gardner's syndrome are highly sensitive to X rays, ultraviolet (UV) rays, MMC, and alkylating agents. Together, these reports suggest the involvement of DNA repair and carcinogenicity at the cellular level in dominant hereditary disease also.

By isolating skin tissue fragments from patients with familial colon polyposis, fibroblasts (CP-S cells) were cultured. When survival rates, based on the colony formation assay method following exposure to X rays and UV rays were compared, CP-S cells showed the same sensitivity as normal human diploid cells. However, there were large individual differences in the sensitivity to MMC. Normal cells were labeled with ^{3}H-thymidine (^{3}H-TdR), a 10-minute process, and then placed at regular time intervals on a membrane filter, and lysed. Short DNA fragments in the earlier stages of DNA synthesis were eluted through a filter, but DNA of large molecular weight remained on the filter (membrane elution

常染色体性劣性様式を示すDNA修復欠損遺伝病に比べて，優性遺伝病における高発癌性の機序は全く分かっていない．Weichselbaumら[7,8]はD-deletion type (13q−)の網膜芽腫患者の皮膚線維芽細胞はX線に高感受性を示すことを報告した．この報告は13染色体長腕の欠損部に，DNA損傷修復に関する遺伝子の存在を示唆している点で極めて重要である．しかし，この報告に反対する結果も報告されているので，[9,10]今後の追試が必要であろう．

大腸腺腫症は大腸の全域に腺腫がびまん性に発生する疾患で，随伴病変の有無により家族性大腸ポリポーシス，Gardner症候群，Turcot症候群に分類される．[11]家族性大腸ポリポーシス及びGardner症候群は，[12]常染色体性優性遺伝疾患であり，極めて高率に大腸癌が発生することと，腫瘍性随伴病変が発生することに特徴がある．[11] Turcot症候群は極めてまれな疾患で，常染色体性劣性遺伝様式を示すことが明らかにされている．[13] Littleら[14]及び他の研究者[15,16]は，家族性大腸ポリポーシス及びGardner症候群の患者から得られた皮膚線維芽細胞がX線，紫外(UV)線，MMC及びアルキル化剤に対して感受性が高いことを報告した．これは優性遺伝病細胞においても，DNA修復と発癌性が関与しているという報告として注目されている．

家族性大腸ポリポーシスと診断された患者から皮膚組織片を分離し，線維芽細胞(CP-S細胞)を培養樹立した．X線及びUV線照射後のコロニー形成法でみた生存率を比較すると，CP-S細胞の感受性は正常ヒト二倍体細胞のそれと同じであった．しかし，MMCに対する感受性には大きな個人差があった．正常細胞を^{3}H-チミジン(^{3}H-TdR)で10分間標識し，定時的に細胞をメンブレン・フィルター上に載せ，細胞を溶解した．DNA合成開始まもない，短いDNA断片はフィルターを通過するが，分子量の大きいDNAはフィルター上に残る(membrane elution法)．

TABLE 1 SOURCES OF ADENOMATOSIS COLI CELLS
表1　大腸腺腫症細胞の由来

Cell Strains 細胞系	Donor 提供者		Remarks 備考
	Age in Years* 年齢	Sex 性	
CP-S3	37	Female 女	Patient has two younger sisters and one younger brother, none of whom have a history of the disease. Her mother (CP-S6) and maternal aunt have familial colon polyposis. Her grandmother died of rectal cancer. 二人の妹と一人の弟がいるが，いずれも発病なし．母親(CP-S6)と叔母が家族性大腸ポリポーシス．祖母は直腸癌で死亡．
CP-S4	46	Female 女	Eldest of three sisters. One of her two sisters has familial colon polyposis and the other, Gardner's syndrome. 三姉妹の長女．二人の妹は，それぞれ家族性大腸ポリポーシスと Gardner 症候群．
CP-S5	48	Female 女	Second eldest of seven brothers and sisters. Two younger brothers have familial colon polyposis, the eldest sister, rectal cancer. 七人兄弟の二女．二人の弟が家族性大腸ポリポーシス．長姉は直腸癌
CP-S6	55	Female 女	Mother of CP-S3. Her elder brother has no history of the disease. One of her two younger sisters has familial colon polyposis. CP-S3 の母親．兄には発病なし．二人の妹のうち，1人は家族性大腸ポリポーシス

**Age at the time of donation of skin tissues.* 皮膚組織を提供したときの年齢．

method). This method was used to compare the elongation pattern of DNA in MMC-treated normal cells, as well as in CP-S cells. DNA elongation was inhibited both in MMC-sensitive cells and in normal cells by MMC at concentrations of more than 0.1 μg/ml. However, MMC-sensitive cells recovered much later than normal cells from DNA elongation inhibition.

MATERIALS AND METHODS

Tissue and Cells

Patients with AC underwent surgery at the Second Department of Surgery, Hiroshima University School of Medicine, and they were all diagnosed as having familial colon polyposis. Skin tissue was collected from the abdomen at operation with the consent of the patient. Table 1 shows the sources from which the cells were derived. For control material, lung fibroblast (LU235) cells were cultured from a 7-month-old infant (male) who died accidentally. Karyotype analysis of the LU235 cells revealed a normal diploid number and no chromosomal aberrations (normal human diploid cells). All cells were subcultured according to a method described previously.[17-19]

Culture Solution

Alpha minimum essential medium (αMEM,

この方法により，MMC処理後の細胞内DNAの伸長パターンを正常細胞及びCP-S細胞について比較した．MMC高感受性細胞及び正常細胞のいずれにおいても，濃度0.1μg/ml 以上の MMC により DNA 鎖の伸長は阻害される．しかし，MMC高感受性細胞の DNA 鎖伸長抑制からの回復は正常細胞のそれに比べて著しく遅れることが分かった．

材料及び方法

組織及び細胞

大腸腺腫症患者は広島大学医学部第二外科で手術を受け，いずれも家族性大腸ポリポーシスと診断された．皮膚組織は患者の同意を得て，手術時に腹部から採集された．細胞の由来は表1に示す．対照実験には，事故で死亡した7か月の乳幼児(男)の肺から線維芽細胞(LU 235)を培養して用いた．LU 235 細胞の核型分析では，異常染色体は全く認められず，正常二倍体性を維持していた．細胞はすべて以前の方法により継代培養した．[17-19]

培養液

本研究で用いたすべての細胞の維持と継代培養には，

GIBCO, USA) culture solution supplemented with 10% fetal bovine serum (FBS, Hy-Clone Co., USA) was used for the maintenance and subculture of all the cells used in this study. During the experiments, cells were grown in αMEM culture medium containing 15% FBS.

X ray Exposure

Cells were exposed to X rays (40 kVp, 5 mA, 0.2 Al filter, and 0.23 mm Al half-value layer) generated by a soft X-ray machine (CMBW-2 Model, Softex Co., Tokyo). The exposure dose rate, determined with a Keithley Condenser Chamber (Keithley Co., USA, sensitivity: 0.937), was 200 R/min. Cells were X-irradiated in a 1 ml cell suspension (1×10^5 cells/ml) uniformly dispersed on 5 cm diameter plastic petri dishes (Falcon Co., USA, Cat. No. 3002) without a cover. After exposure, an appropriate number of cells were inoculated in dishes and cultured at 37°C in a 5% CO_2 incubator. After a 2-week incubation period, counts were made for each colony and dose-survival response was assessed.

Exposure to Ultraviolet Rays

Petri dishes (5 cm) were inoculated with an appropriate number of cells, so that after taking into account cell plating efficiency and cell killing following UV ray treatment, a few of the cells would survive to grow into macroscopic colonies. Culture medium was removed and 0.25 ml of negative phosphate buffer saline (PBS(−)) solution was added to prevent the cells from drying and followed with UV ray exposure (Toshiba GL15, Toshiba, Tokyo). The exposure dose rate was determined with a Topcon UV radiometer (UVR254, Topcon Co., Tokyo). The exposures were made at dose rates of 2 J/m^2·sec or 3 J/m^2·sec, with no differences being noted between the two by dose rate. After exposure, 5 ml of culture solution (αMEM, 15% FBS) was added to the dishes. Dose-survival relationship was evaluated by the colony formation assay method.

Cytotoxicity of Mitomycin-C

A suitable number of cells to form distinct colonies after treatment were added to the petri dishes (5 cm), and 18-20 hours later the culture medium (αMEM, 15% FBS) was removed. Then αMEM culture solution (containing no serum) with various concentrations of MMC (P-L Biochemicals, Inc., USA) was added to the dishes and the cells were cultured at 37°C in

10％の牛胎血清(Hy-Clone 社，米国)を添加したαMEM(GIBCO 社，米国)培養液を用いた．すべての実験には，αMEM 培地に15%の牛胎血清を添加した．

X線照射

軟X線発生装置(CMBW-2 Model, Softex 社，東京)から発生するX線(40kVp, 5 mA, 0.2Al フィルター，0.23mm Al 半価層)を細胞に照射した．Keithley Condenser Chamber(Keithley 社，米国，感度0.937)で測定した照射線量率は200R/min であった．1 ml の細胞浮遊液(1×10^5個/ml)を直径 5 cmのプラスチックペトリ皿(Falcon 社，米国，Cat. No. 3002)に均一に分散し，ふたを取り除いてX線を照射した．照射後，適当な細胞数をペトリ皿に播種し，37°C，5％ CO_2 条件下で培養した．培養 2 週間後にコロニー数を計数し，線量一生存率関係を求めた．

紫外線照射

細胞の効果的播種とUV線照射後の細胞死滅を計算に入れて，幾つかの細胞が肉眼的なコロニーに成長するように，適当数の細胞をペトリ皿(径 5 cm)に播種した．細胞の乾燥を防ぐために0.25ml の PBS(−)緩衝液を添加後，UV線(Toshiba GL15，東芝，東京)を照射した．線量率はTopcon UV radiometer(UVR254，Topcon 社，東京)で測定した．2J/m^2·sec 又は3J/m^2·sec の線量率で照射したが，両者の線量率の間に全く差はなかった．照射後，培養液(αMEM，15% FBS) 5 ml をペトリ皿に添加した．コロニー形成法によって線量一生存率関係を求めた．

マイトマイシン-Cの細胞毒性

処理後にコロニー形成ができる程度の適当数の細胞をペトリ皿(径 5 cm)に播種し，18～20時間後に培養液(αMEM，15% FBS)を捨てた．種々の濃度のMMC(P-L Biochemicals 社，米国)を含むαMEM培養液(血清を含まず)をそのペトリ皿に添加し，細胞を37°C，5％ CO_2 条件下で培養した．1時間

5% CO_2. One hour later, the cells were washed twice in Eagle's MEM (Nissui Pharmaceuticals, Tokyo) solution containing no serum. After adding 5 ml of the complete culture medium (αMEM + 15% FBS), the concentration-survival relationship of MMC was evaluated by the colony formation assay method.

Evaluation of the Elongation Pattern of Newly Synthesized DNA (Membrane Elution Method)

Plastic bottles (Corning Co., USA, Cat. No. 25100) having a cell growing surface area of 25 cm^2 were plated with 2.5×10^5 cells and cultured at 37°C. After 24 hours, the cells were exposed for 6 hours to medium containing ^{14}C-thymidine (^{14}C-TdR, 0.02 μCi/ml, 22.8 μCi/mmole, Amersham, UK). Eighteen hours later they were exposed for 10 minutes to culture medium containing ^{3}H-TdR (10 μCi/ml, 1.06 μCi/mmole, Amersham, UK) for labeling. Then the cells were harvested with 0.25% trypsin (1:250, Difco, USA) + 0.02% EDTA solution at various times, and 5×10^5 cells were placed on a membrane filter (2.0 μm pore size, Millipore Co., USA, Cat. No. BSWPO2500) and washed with 10 ml of PBS(−) buffer solution. Three milliliters of a cell-lysing solution (2% SDS+0.025 M H_2-EDTA, pH 10.0) was poured on the cells attached to the filter. Upon efflux of the cell-lysing solution from the filter, 2 ml of cell-lysing solution containing proteinase K (0.5 mg/ml, Merck Co., USA) was slowly poured on the cells. After efflux of the proteinase K solution, 3 ml of washing solution (0.02 M Na_2-EDTA, pH 10.0) was poured on the filter. The cell-lysing solution, proteinase K solution, and washing solution were collected as eluate. Using a liquid scintillation counter (Aloka LSC-670, USA), radioactivity levels of the eluate and of the filter were determined.

Evaluation of DNA Elongation Inhibition by MMC

The same plating and culturing methods described above were used. After 45 hours, αMEM culture medium, without serum but containing MMC at various concentrations, was added to the cells for 1 hour at 37°C in 5% CO_2. After two hours, cells were labeled with ^{3}H-TdR for 10 minutes. The cells were then detached at various time intervals and the degree of DNA elongation of the newly synthesized DNA was determined by the membrane elution method described above.

後，血清を含まないEagle MEM(日水製薬，東京)で2回洗浄した．完全培養液(αMEM＋15% FBS)5 ml を添加後，コロニー形成法でMMCの濃度ー生存率関係を求めた．

新しく合成される DNA鎖の伸長パターンの評価 (Membrane Elution 法)

2.5×10^5 個の細胞を，培養面積25cm^2 のプラスチックボトル(Corning 社，米国 Cat. No. 25100)に播種し37°Cで培養した．24時間後に，^{14}C-チミジン(^{14}C-TdR, 0.02μCi/ml, 22.8μCi/mmole, Amersham 社，英国)を含む培養液中で6時間細胞を培養した．18時間後に，^{3}H-TdR(10μCi/ml, 1.06μCi/mmole, Amersham 社，英国)を含む培養液中で10分間標識した．標識後，0.25%トリプシン(1: 250, Difco 社，米国)＋0.02% EDTA 培養液で経時的に細胞を剝離した．5×10^5 個の細胞を，メンブレンフィルター(2.0μm の孔，Millipore 社，米国，Cat. No. BSWPO 2500)上に載せ，PBS(−)緩衝液10ml で洗浄した．フィルター上の細胞に細胞融解液(2% SDS＋0.025 M H_2-EDTA, pH 10.0) 3 ml を注いだ．細胞融解液がフィルターから流出した後，プロティナーゼK (0.5mg/ml, Merck 社，米国)を含む細胞融解液2ml を細胞の上に静かに注いだ．プロティナーゼK溶液の流出後，洗浄液(0.02 M Na_2-EDTA, pH 10.0) 3ml をフィルター上に注いだ．細胞融解液，プロティナーゼK溶液及び洗浄液を集めて流出液とした．流出液中及びフィルター上の放射能活性を液体シンチレーションカウンター(Aloka LSC-670, 米国)で測定した．

MMC による DNA鎖伸長阻害の評価

播種及び培養法は前記のとおりである．培養45時間後に，種々の濃度の MMC を含むαMEM 培養液(血清を含まず)を添加し，37°C，5% CO_2 条件下で1時間培養した．2時間後に細胞は ^{3}H-TdR で10分間標識した．以後，経時的に細胞を剝離し，前述の membrane elution 法により，新しく合成される DNA の伸長度を測定，比較した．

RESULTS

Sensitivity to Radiation and MMC of Cells Derived from Patients with Familial Colon Polyposis

Figures 1-3 show the respective effects of exposure to X rays, UV rays, and MMC on the survival of CP-S cells, as determined by the colony formation assay method. In each figure, plating efficiencies by colony formation assay were greater than 15% for nonirradiated and non-MMC-treated cells. The means and standard deviations from at least four experimental trials are shown. X-ray sensitivity of CP-S cells and normal human diploid LU235 cells were similar. The n value (value extrapolated from the linear portion of the survival curve, Figure 1, to the vertical axis) was 1.8 and the D_0 (dose corresponding to the inverse of the slope determined from the linear portion of the survival curve resulting in 37% survival) was 117 R.

After UV ray exposure, LU235 cells and CP-S4 cells both showed the same n values of 1.2 and D_0 values of 4.75 J, while CP-S3 cells and CP-S5 cells both showed the same n values of 1.2 and D_0 values of 4.0 J. There appeared to be no statistically significant differences between the two groups.

However, large individual differences were observed in MMC sensitivity of CP-S cells. CP-S4 cells showed the same MMC sensitivity as LU235 cells, while CP-S5 cells were much more sensitive. The CP-S3 cells sensitivity to MMC was between normal and the CP-S5 cells sensitivity. Also the CP-S6 cells were slightly more sensitive than the normal LU235 cells.

Elongation Pattern of Newly Synthesized DNA

The pattern of DNA elongation was assessed by membrane elution method. Figure 4 shows the results of two tests using LU235 cells. Because the molecular weight of ^{14}C-TdR-labeled DNA is large, about 98% of the DNA molecules remained on the filter. DNA labeled with ^{3}H-TdR consisted of molecules in the process of DNA synthesis and small synthesized DNA fragments. Fragments with small molecular weight were easily eluted through the filter (pore size: 2.0 μm). LU235 cells in the 10th-15th generation after establishing the culture, proliferated with a doubling time of 20 to 24 hours. Immediately after ^{3}H-TdR labeling, 65%-70% of the new DNA was eluted through

結　果

家族性大腸ポリポーシス患者由来細胞の放射線及びMMCに対する感受性

CP-S細胞のX線，UV線及びMMCに対する生存率効果を，コロニー形成法でみた結果を各々図1－3に示す．各図において，放射線非照射群及びMMC非処理群の細胞のコロニー形成率は15%以上であった．それぞれ4回以上の実験結果の平均と標準偏差で表した．CP-S細胞のX線感受性はヒト正常二倍体細胞(LU235)のそれと全く同じである．n値(図1，直線部分の縦軸への外挿値)が1.8，D_0(37%生存率を与える直線部分から求めた勾配の逆数で表した線量値)は117Rであった．

UV線照射後のLU235細胞及びCP-S4細胞のn値は1.2，D_0は4.75Jで，CP-S3細胞及びCP-S5細胞のn値は1.2，D_0は4.0Jであった．両者の間に統計的な差異はないと思われる．

CP-S細胞のMMCに対する感受性には大きな個人差があった．CP-S4細胞はLU235細胞と全く同じ感受性を示すが，CP-S5細胞は非常に高い感受性を示した．CP-S3細胞は正常細胞とCP-S5細胞のほぼ中間の感受性をもつ．CP-S6細胞もLU235正常細胞よりやや高い感受性を示した．

新しく合成されるDNAの伸長パターン

DNA鎖の伸長パターンをmembrane elution法で評価した．LU235細胞を用いての2回の実験結果を図4に示す．^{14}C-TdRで標識されたDNAは分子量が大きいために，フィルターを通過することなく，DNA分子の約98%がフィルター上に残った．^{3}H-TdRで標識されたDNAには，DNA合成中の分子，あるいはDNA合成開始後の小さな断片が含まれる．分子量の小さい断片はフィルター(2.0μmの孔)を容易に通過した．ここで用いたLU235細胞は培養樹立後10－15世代目で，倍加時間が20－24時間であった．^{3}H-TdR標識直後には，65%－70%の新生DNAが

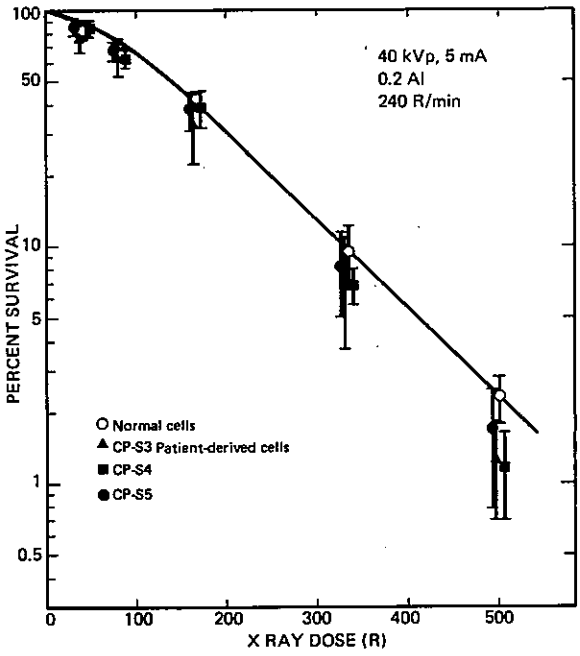

Figure 1. Dose-survival relationship of normal human diploid LU235 cells and familial colon polyposis patient-derived cells following exposure to various X-ray doses. The bars indicate for each cell the mean and standard deviation of the results of four or more tests. The value extrapolated onto the longitudinal axis from the linear portion (n value) is 1.8, and the dose based on the linear portion (D_0) corresponding to a survival rate of 37% is 117 R.

図1　種々の線量のX線照射後の，ヒト正常二倍体細胞と家族性大腸ポリポーシス患者由来細胞の線量一生存率関係．棒は各細胞について，4回以上の実験結果の平均と標準偏差を示す．直線部分の縦軸への外挿値（n値）は1.8，直線部分の37%生存率を与える線量（D_0）は117Rである．

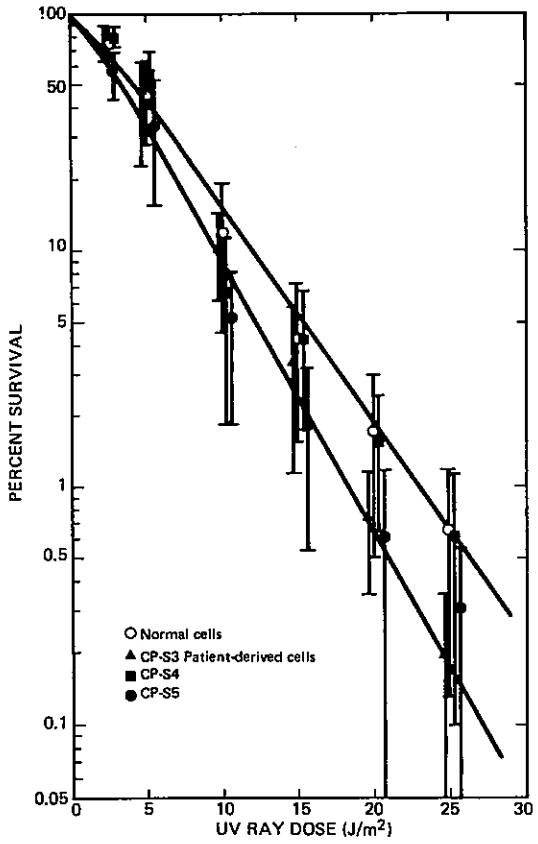

Figure 2. Dose-survival relationship of normal cells and familial colon polyposis patient-derived cells following exposure to various UV ray doses. The bars indicate for each cell the mean and standard deviation of the results of four or more tests. The n value is 1.2 for all of the four types of cells. D_0 is 4.75 J for normal cells and CP-S4 cells, and 4.0 J for CP-S3 and CP-S5 cells.

図2　種々の線量のUV線照射後の，正常細胞と家族性大腸ポリポーシス患者由来細胞の線量一生存率関係．棒は各細胞について4回以上の実験結果の平均と標準偏差を示す．4種の細胞ともn値は1.2である．D_0は正常細胞及びCP-S4細胞が4.75J，CP-S3細胞とCP-S5細胞が4.0Jである．

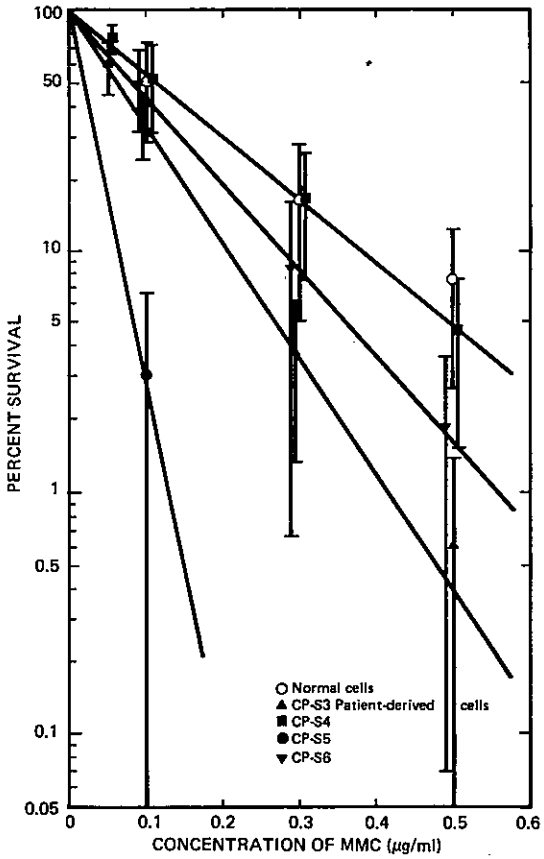

Figure 3. Concentration-survival relationship of normal cells and familial colon polyposis patient-derived cells treated with MMC at various concentrations. The bars indicate for each cell the mean and standard deviation of the results of four or more tests. MMC was dissolved in a culture solution containing no serum, and the cells were treated with this for one hour at 37°C.

図3　種々の濃度のMMC処理後の，正常細胞と家族性大腸ポリポーシス患者由来細胞の濃度ー生存率関係．棒は各細胞について4回以上の実験結果の平均と標準偏差を示す．MMCは無血清培養液に溶解して，これで細胞を37°Cで1時間処理した．

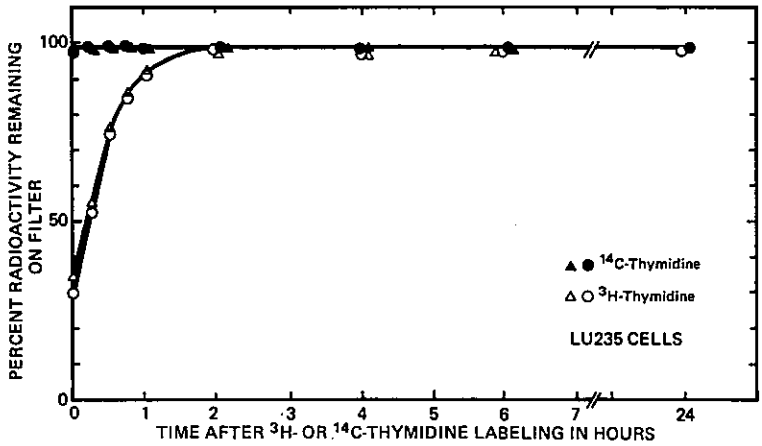

Figure 4. Percent radioactivity remaining on filter with time following ^{3}H-TdR labeling and after the cells were lysed and washed on the filter (2.0 μm pore size). The cells were cultured in advance in a culture solution containing ^{14}C-TdR (0.02 μCi/ml) for six hours. After 18 hours, the cells for labeling were cultured in a culture solution containing ^{3}H-TdR (10 μCi/ml) for 10 minutes. The figure shows the results of two tests using normal human diploid LU235 cells.

図4　^{3}H-TdR標識後，経時的に細胞をフィルター（2.0μmの孔）上で融解し，洗浄後フィルター上に残った放射能活性の割合．細胞はあらかじめ^{14}C-TdR（0.02μCi/ml）を含む培養液中で6時間培養した．標識のための細胞を18時間後に，^{3}H-TdR（10μCi/ml）を含む培養液中で10分間培養した．ヒト正常二倍体LU 235細胞を用いての2回の実験結果を示す．

the filter. After one hour, more than 90% of the DNA remained on the filter. After two hours, the percent radioactivity of ^{3}H-TdR on the filter was identical with that of ^{14}C-TdR on the filter.

Inhibition of DNA Elongation by MMC and Recovery from Inhibition

After MMC treatment, DNA elongation of cells differing in their sensitivity to MMC were compared using the membrane elution method (Figure 5). DNA elongation in normal (LU235 cells) and MMC sensitive cells (CP-S3 and CP-S5 cells) was inhibited by MMC at concentrations greater than 0.1 μg/ml. Inhibition of MMC-sensitive cells was greater and its recovery much slower compared to normal LU235 and CP-S4 cells. Two hours after ^{3}H-TdR labeling, DNA synthesis of normal LU235 and CP-S4 cells treated with MMC at concentrations up to 10 μg/ml was recovered the same as that in nontreated cells. However, MMC-sensitive cells, treated with MMC at a concentration of 10 μg/ml, continued to show DNA synthesis inhibition.

Figure 6 compares DNA elongation of the 50 μg/ml MMC-treated group shown in Figure 5 with the MMC-nontreated controls. Inhibition by MMC of DNA elongation in normal LU235 and CP-S4 cells decreased rapidly with the culturing time of the cells. The recovery patterns of both cell types were identical: at two hours after treatment, DNA synthesis recovery was to about 80% of that for cells not treated with MMC. However, MMC-sensitive cells showed only slight recovery during the first hour after the culture was initiated. After two hours, there was only 40%-60% recovery compared with the MMC-nontreated controls.

DISCUSSION

AC is a general term for diseases in which many polyps of diffuse adenoma develop in the large intestine.[11] Such patients develop cancer of the large intestine at a relatively high frequency. Of the diseases listed as AC, the primary one is familial colon polyposis, an autosomal dominant hereditary diseases.[11,20] Others include Gardner's syndrome (an autosomal dominant hereditary disease) and Turcot's syndrome (an autosomal recessive hereditary disease). A number of studies have reported on the frequency of AC.

フィルターを通過した．1時間後には90%以上のDNAがフィルターに残った．2時間後には，フィルター上の ^{3}H-TdR 及び ^{14}C-TdR の放射活性の割合は全く一致するようになった．

MMCによるDNA鎖伸長の阻害と回復

Membrane elution 法を使って，MMC感受性の異なる細胞における，MMC処理後のDNA鎖伸長を比較した（図5）．正常細胞（LU235）及びMMC高感受性細胞（CP-S3，CP-S5）のいずれにおいても，DNA鎖伸長は濃度0.1μg/ml 以上のMMCによって抑制された．正常 LU235細胞及び CP-S4 細胞に比べて，MMC高感受性細胞における DNA 鎖伸長の抑制は大きく，その回復も非常に遅れた．^{3}H-TdR 標識2時間後には，正常 LU235 細胞及び CP-S4 細胞では，10μg/ml 濃度までの MMC 処理群の DNA 合成が MMC 無処理細胞とほぼ同程度まで回復している．しかし，MMC高感受性細胞においては，10μg/ml 濃度の MMC 処理群では，DNA 合成の抑制がまだはっきりと認められる．

図5の50μg/ml MMC を処理した場合の DNA 鎖伸長度を MMC 無処理対照群のそれと比較したのが図6である．正常LU235細胞及びCP-S4 細胞での MMC による DNA 鎖伸長抑制は，培養時間の経過とともに速やかに回復した．両者の回復パターンは完全に一致する．2時間後には，MMC無処理細胞群の DNA 合成の約80%まで回復した．しかし，MMC高感受性細胞においては，培養1時間目までは回復が少なく，2時間後には MMC 無処理対照群と比較して40%－60%までの回復しかみられなかった．

考　察

大腸腺腫症は，大腸の全域に多数の腺腫がびまん性に発生し，[11] 極めて高率に大腸癌が発生する疾患の総称である．この中の主な疾患は家族性大腸ポリポーシスであり，これは常染色体性優性遺伝疾患である．[11,20] ほかに Gardner 症候群（常染色体性優性遺伝疾患）と Turcot 症候群（常染色体性劣性遺伝疾患）がある．大腸腺腫症の頻度については幾つかの

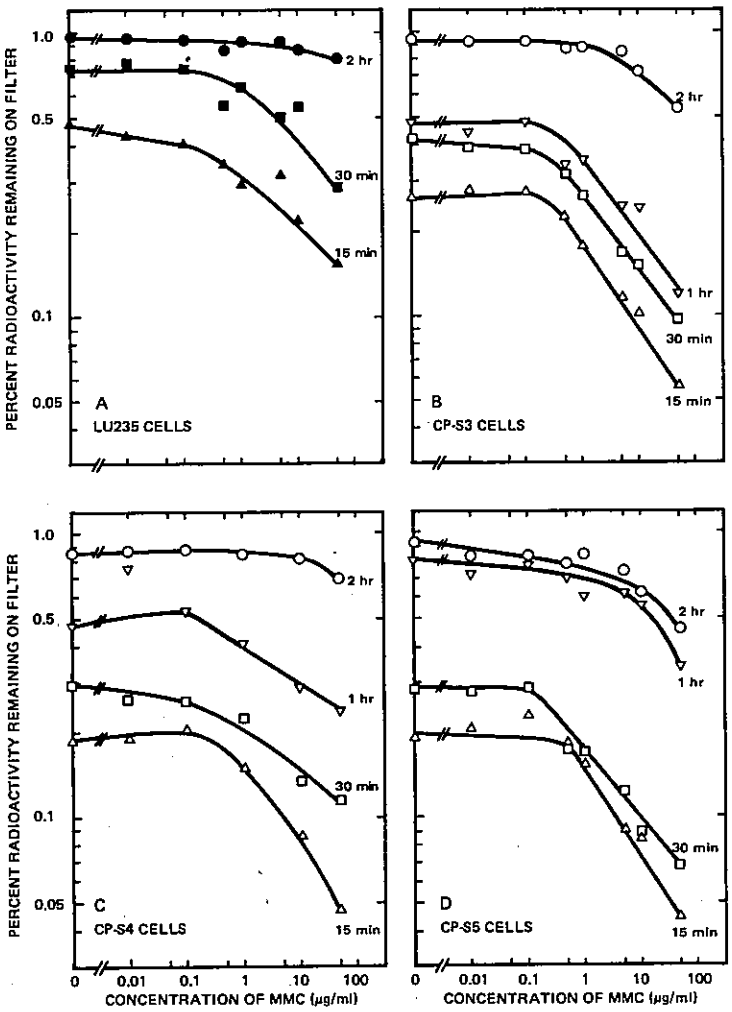

Figure 5. Percent radioactivity remaining on the filter when MMC-treated cells, allowed to incorporate ^{3}H-TdR, were lysed on the filter at certain time intervals. The cells were treated for one hour with MMC at various concentrations, and two hours thereafter, labeled with ^{3}H-TdR (10 μCi/ml, culturing a 10-minute process). The time given in the figures indicates culturing time between ^{3}H-TdR labeling and cell detachment.

図5　MMC処理した細胞に ^{3}H-TdR を取り込ませ，経時的に細胞をフィルター上で融解したときの，フィルター上に残る放射能活性の割合．細胞は，種々の MMC 濃度で1時間処理し，2時間後に ^{3}H-TdR (10μCi/ml) で10分間標識した．図中の時間は ^{3}H-TdR 標識から細胞剝離までの培養時間を示す．

Reed and Neel,[21] and Pierce[22] reported the frequency in the United States to be 1.21×10^{-4} and 1.46×10^{-4}, respectively. While in England, Veale[23] reported an AC frequency of 0.42×10^{-4}. According to Murata et al,[24] the frequency in Japan is 5.74×10^{-5}, and while indicating that the number of cases was small, regional differences in frequency in Japan were thought to be virtually absent. A remarkably high frequency of

報告がなされている．Reed と Neel[21] 及び Pierce[22] は，米国における頻度はそれぞれ 1.21×10^{-4} 及び 1.46×10^{-4} であると報告している．Veale[23] は英国での頻度は 0.42×10^{-4} であると報告している．日本における頻度は，Murata ら[24] が 5.74×10^{-5} であると報告した．また彼らは例数が少ないとことわりながらも，日本国内における発生頻度に地域差はまずないであろうと推測している．本研究に協力

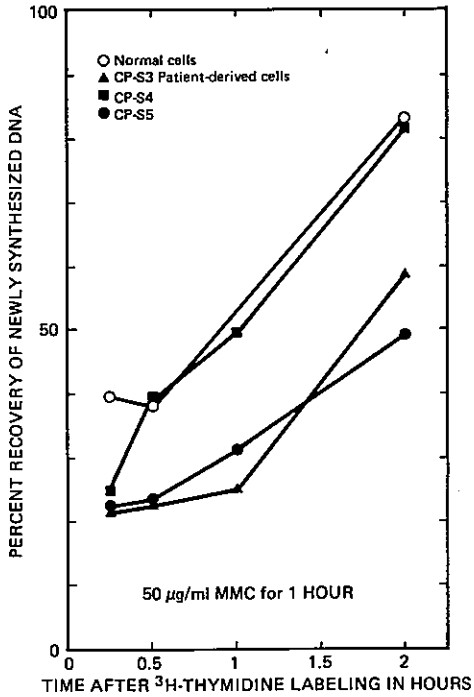

Figure 6. Results of calculations based on Figure 5, indicating the ratio of radioactivity of cells treated with 50 μg/ml MMC and remaining on the filter after being lysed there, to that of MMC-nontreated cells.

図 6　50μg/ml 濃度の MMC 処理細胞をフィルター上で融解し，フィルター上に残った放射能活性を，MMC 無処理を用いた場合のそれに照してみた割合を図 5 から計算した結果

AC has been observed in families of patients participating in this study (Table 1), thus supporting the assumption that it is a genetically transmitted disease.

It is well known that ionizing radiation, UV rays, and MMC induce different types of DNA damage in exposed cells. X rays usually induce DNA strand breaks and damage to the DNA bases. UV radiation forms pyrimidine dimers efficiently, particularly thymidine dimers. MMC primarily induces cross-linking of DNA and modification of the DNA bases. Examination of lethal effects on cells from DNA damage should provide useful information for studying DNA metabolism in AC cells. CP-S cells demonstrated sensitivity to X rays (Figure 1) and UV rays (Figure 2) quite identical with that of normal cells. However, a large difference was observed in the sensitivity of cells to MMC between individuals (Figure 3): CP-S4 cells (Table 1) showed the same sensitivity to MMC as normal cells, but CP-S5 cells exhibited greater sensitivity to MMC. The donors of CP-S3 cells and CP-S6 cells were related (mother and child), and each showed an elevated sensitivity to MMC.

された患者の家族内には，非常に多数の大腸腺腫症が観察されており（表 1），本症が遺伝要因の強い疾患であることを裏付けている．

電離放射線，UV 線及び MMC は，被曝した細胞にそれぞれ異なった DNA 損傷を誘起することはよく知られている．X 線は DNA 鎖切断あるいは DNA 塩基の損傷を主に引き起こす．UV 線はピリミジンダイマー，特にチミジンダイマーを効率よく形成する．MMC は DNA クロスリンキング及び DNA 塩基の修飾を主に誘起する．これらの DNA 損傷の細胞致死効果を調べることは，大腸腺腫症細胞内における DNA 代謝を調べる上で有用な情報を提供してくれるであろう．CP-S 細胞は X 線（図 1）及び UV 線（図 2）に対して，正常細胞と全く同じ感受性を示した．ところが，MMC に対する感受性には大きな個人差がみられた（図 3）．CP-S4 細胞（表 1）は正常細胞と全く同じ MMC 感受性を示すが，CP-S5 細胞は極めて大きな MMC 高感受性を示した．CP-S3 細胞と CP-S6 細胞の提供者は母子関係にあり，いずれも MMC 感受性はやや高い．

Using CP-S cells and normal cells which differ in their sensitivity to MMC, the elongation of newly synthesized DNA after treatment with MMC was compared by measuring the quantity of DNA, which could be eluted through a 2.0 μm pore filter, at various time intervals after DNA synthesis began (membrane elution method). Although errors might occur because of pore size differences between filters, such errors were minimized by using filters from the same manufacturer's lot. In this study, DNA elongation patterns were examined, beginning two hours after MMC treatment. When the DNA elongation patterns were examined immediately after MMC treatment, wide variations were observed between trials. This variation probably occurred either because the MMC treatment was conducted in a culture solution containing no serum, or because the cells were washed twice after MMC treatment. Preliminary experiments showed that even if cells, exposed to ^{14}C-TdR (0.02 μCi/ml) for 5 hours beginning 24 hours after plating, were treated with MMC at concentrations ranging from 0-50 μg/ml, more than 95% of the radioactivity remained on the filter until the fourth hour after MMC treatment. In other words, destruction of DNA was not detected by the membrane elution method until four hours after MMC treatment.

MMC感受性のそれぞれ異なるCP-S細胞と正常細胞を用いて，MMC処理後に新しく合成されるDNAの伸長度を比較した．すなわち，2.0μmの孔のフィルターを通過できる大きさのDNA量を，DNA合成開始から経時的に比較した（membrane elution法）．この方法は，フィルターのロットによってわずかの誤差を生じるが，製造社の同一ロットのフィルターを使うことによりその誤差を最小限にした．本研究では，MMC処理後2時間目からのDNA鎖伸長パターンを調べた．MMC処理直後にDNA鎖伸長パターンを調べると，実験ごとに大きなばらつきがみられた．その理由はMMC処理は無血清培養液中で行っていること，あるいはMMC処理後細胞を2回洗浄する操作が入ることなどによると思われる．細胞播種後24時間目から^{14}C-TdR（0.02μCi/ml）を6時間取り込ませた細胞に濃度0－50μg/mlのMMCを処理しても，MMC処理後4時間目までは95%以上の放射能活性がフィルター上に残ることは予備実験で確認した．すなわち，MMC処理後4時間目までは，DNAの破壊はmembrane elution法では検出されなかった．

Both cells highly sensitive to MMC and normal cells showed similar DNA elongation inhibition when the concentration of MMC was more than 0.1 μg/ml (Figure 5). However, in the sensitive cells, inhibition of DNA elongation was substantially greater at higher MMC concentrations (Figures 5 and 6). The amount of DNA from normal and CP-S4 cells remaining on the filter increased with time after ^{3}H-TdR labeling; however, the recovery of DNA elongation in MMC-sensitive cells was delayed (Figure 6). A significant difference in lethal effects of MMC was observed between CP-S3 cells and CP-S5 cells (Figure 3); although, there was no difference either in the pattern of DNA elongation inhibition by MMC or in the pattern of recovery. This may be because the degree of DNA elongation determined by the membrane elution method is based on whether DNA was small enough to pass through 2.0 μm size pores. In contrast, many factors other than DNA synthesis inhibition are considered to be involved in the lethal effects of MMC.

MMC高感受性細胞及び正常細胞のいずれにおいても濃度0.1μg/ml以上のMMC処理ではDNA鎖伸長の抑制に差は見られなかった（図5）．MMC高感受性細胞の高濃度におけるMMC処理によるDNA鎖伸長の抑制は顕著であった（図5及び6）．正常細胞及びCP-S4細胞では，^{3}H-TdR標識後の時間経過に伴って，フィルター上に残るDNA量が多くなる．しかし，MMC高感受性細胞においてはDNA鎖伸長の回復は著しく遅れた（図6）．MMCの致死効果には，CP-S3細胞とCP-S5細胞の間に明確な差がみられた（図3）．しかし，MMCによるDNA鎖伸長の抑制とその回復パターンには，CP-S3細胞とCP-S5細胞との間に差は認められなかった．この理由は，membrane elution法でみるDNA鎖伸長度の判定規準が，DNAが2.0μmの孔のフィルターを通過できる大きさか否かにかかわっている．それに対比してDNA合成抑制以外に，多くの要因がMMCの致死効果に含まれるであろう．

The membrane elution method used in this study is an application of the alkaline elution method developed by Kohn et al.[25,26] And this is the first report in which the method is used as a means of evaluating DNA elongation. This method involves the important question of what molecular weight of DNA is sufficient to cause DNA to remain on the filter. Kohn et al[25,26] and Suzuki et al[27] reported that even small DNA fragments generated by exposure to high doses of ionizing radiation (~1,000 R) are not eluted through the filter in the process of cell lysis and washing. That is, DNA eluted through the filter by a pH 10.0 cell lysis solution, proteinase K solution, and washing solution are fragments of small molecular weight which have just started DNA synthesis. Accordingly, it is believed that in the case of cells which are highly sensitive to MMC, such as those used in this study, the DNA synthesis mechanism has MMC-sensitive sites involved in the initial stages of DNA synthesis.

AC is important as a model in studying the inheritance of proneness to cancer. If the cancer proneness of the disease is to be closely related to somatic cell mutation, investigation of primary cultures of skin fibroblasts from such patients should be useful in the exploration of various features of dominant tumor-forming mutants in relation to the mechanism of carcinogenesis.

本研究での membrane elution 法は，Kohn ら[25,26]の開発した alkaline elution 法を応用したものであるが，DNA 鎖伸長度の評価法としては本報告が最初である．この方法では，どの程度の大きさの分子量をもつ DNA がフィルター上に残るようになるのかという，今後検討されなければならない重大な問題点を含んでいる．Kohn ら[25,26]あるいは鈴木ら[27]によると，高線量（~1,000R）の電離放射線照射によって生じた小さな DNA 断片でも，細胞融解及び洗浄中にはほとんどフィルターを通過しない．すなわち，pH 10.0 の細胞融解液とプロティナーゼK溶液，洗浄液でフィルターから流出する DNA は，DNA 合成開始直後の非常に分子量の小さい断片である．したがって，本研究で用いた MMC 高感受性細胞の DNA 合成機構は，MMC に感受性の高い部位を DNA 合成開始期にもつことが予想される．

ともあれ，大腸腺腫症は遺伝と癌との関係を結び付ける重要なモデルであると考えられている．本症の高発癌性が体細胞突然変異と密接な関係があるとするならば，優性に表現される造腫瘍性突然変異体の実体，ひいてはその発癌機構を探る上で，本症患者の皮膚線維芽細胞の初代培養樹立は，有用な情報を提供してくれると思われる．

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