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ヒト皮膚線維芽細胞の初代培養における X線誘発損傷からの回復

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SUMMARY

Human skin fibroblast cells from six patients were obtained during surgical operations and grown in culture. Dose-response survival curves from single dose exposures of X rays were developed for the six cell strains. Individual D_0 values* varied in the six strains from 61 to 83 cGy. The shouldered survival curves had extrapolation number (n) values* ranging from 2.2 to 4.8. To assess repair of sublethal damage, cells were exposed to a total dose of 304 cGy split into two equal fractions separated by varying time intervals. Maximal increase in cell survival was observed when the time interval was at least three hours. Dose-response curves were generated for the six cell strains by first irradiating cells with 152 cGy X rays and then allowing four hours for recovery from sublethal damage before exposing them to second graded doses. The fractionated dose-response survival curves were distinctly different from the single dose exposure curves and confirmed the ability of these cells to recover from X-ray-induced damage.

要 約

外科手術を受けた6人の患者のヒト皮膚線維芽細胞を入手し培養した．六つの細胞株について，X線の単一線量照射の線量反応生存曲線を作成した．六つの細胞株では，個々の D_0 値*は61cGy から83cGyまでに及んだ．肩状を呈する生存曲線は，2.2から4.8までの補外数(n)値*を示した．亜致死性損傷の修復を評価するために，304cGy の合計線量を2等分し，種々の時間間隔で細胞に照射した．細胞の生存率は，時間間隔が少なくとも3時間の場合に最大となった．また，最初 152cGy のX線照射を行い，4時間培養して亜致死性損傷から回復させた後，2回目の照射として種々の線量を与えた場合の線量反応曲線をこれら六つの細胞株について描いた．線量を2回に分割した場合の線量反応生存曲線は，1回照射の曲線と著しく異なり，これらの細胞にX線誘発による損傷からの回復能力があることを確認した．

D_0 – the dose required to reduce survival to 0.37 in the exponential region of the survival curve; n – the extrapolation number, the value obtained by extrapolating the exponential portion of the survival curve to the zero dose intercept. 1 Gray (Gy) = 100 cGy = 100 rad

D_0 – 生存曲線の指数関数の領域における生存率を0.37まで低下させるに必要な線量値; n – 補外数，すなわちゼロ線量切片に対する生存曲線の指数関数部分を補外することにより得た値．1 Gray = 1 Gy = 100cGy = 100rad

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INTRODUCTION

Human fibroblasts, either grown in primary culture or as established cell lines, have been used for years as a model of normal tissue response to radiation exposure. Several investigators have examined the repair capacity of human fibroblasts and have reported the inability of human fibroblasts to repair sublethal radiation damage. Using cell survival as a measure of effect, Puck et al,[1] Norris and Hood,[2] and Weichselbaum et al[3] found that cultured diploid human fibroblast dose-response survival curves were unshouldered and exponential throughout the dose exposures. However, other investigators used more sensitive techniques to elucidate the potential for repair of damage. Cox and Masson[4] found that HF-10 and CL15 diploid human fibroblast cultures were unshouldered in early cultures (6-25 generations) but became shouldered beyond 35 generations. Deschavanne et al[5], Dutreix et al,[6] and Arcangeli et al[7] used large radiation dose fractions and found repair of sublethal damage that was not observed with small fractions. Malcolm and Little[8] used exponentially growing and plateau phase cultures to show rapid recovery in human fibroblast cells. Freeman et al[9] exposed human fibroblast cells to single doses, multiple fractions of 56 cGy, or two different dose rates of X rays and showed significant β terms (linear-quadratic dose-effect model of Kellerer and Rossi[10]) suggesting the accumulation of sublethal damage. They subsequently showed that repair of sublethal damage had occurred.

The present study examines the dose-response curves of fresh cultures of human skin fibroblast cells irradiated with X rays. Dose fractionation was used to elucidate the repair capacity of the human cells.

MATERIALS AND METHODS

Cell Culture and Irradiation

Skin tissue samples were obtained from six female patients from 52 to 57 years of age during surgery at the Second Dapartment of Surgery, Hiroshima University School of Medicine. Excised tissues were immediately placed in sterile containers surrounded by ice and transported to the laboratory. After removal of fatty tissue, the samples were minced into small pieces and pressed onto Falcon plastic petri dishes containing αMEM medium with 15% fetal bovine serum and antibiotics (penicillin, 100 IU/ml and strepto-

緒　言

初代培養において増殖させるか，又は細胞株として樹立したヒト線維芽細胞は，放射線被曝に対する正常組織の反応のモデルとして長年使用されてきた．数人の研究者がヒト線維芽細胞の修復能を調査し，ヒト線維芽細胞が亜致死性損傷を修復する能力をもたないことを報告している．Puck ら，[1] Norris 及び Hood，[2] Weichselbaum ら[3] は，影響の測定に細胞生存率を用い，培養したヒト二倍体線維芽細胞の線量反応生存曲線は肩状を呈さず，いずれの照射線量においても指数関数的であることを観察した．しかし他の研究者は，損傷に対する修復の可能性を明らかにするために更に高感度の技法を用いた．Cox 及び Masson[4] は，HF-10及び CL15ヒト二倍体線維芽細胞の培養では，初期（6-25世代）の生存曲線は肩状を呈さないが35世代以降には肩状を呈することを観察した．Deschavanne ら，[5] Dutreix ら，[6] 及び Arcangeli ら[7] は放射線量の分割を多くした場合，分割が少ない場合には認められなかった亜致死性損傷の修復を観察した．Malcolm 及び Little[8] は，ヒト線維芽細胞の急速な修復を示すために，指数関数的に増殖中及び，プラトー期に達した培養系を用いた．Freeman ら[9] は，ヒト線維芽細胞にX線の単一線量，56cGy を何回も照射，又は二つの異なる線量率で照射し，亜致死性損傷の蓄積を示唆する有意な β 項（Kellerer 及び Rossi[10] の線形２次線量効果モデル）を示した．彼らはその後，亜致死性損傷の修復が行われたことを示した．

本研究では，X線を照射したヒト皮膚線維芽細胞の新鮮な培養系の線量反応曲線を検討する．ヒトの細胞の修復能力を究明するために，線量の分割を行った．

材料及び方法

細胞培養及び放射線照射

広島大学医学部第二外科学教室で，52～57歳の女性患者６例から，外科手術により皮膚組織標本が得られた．切除した組織は直ちに氷中の無菌容器に入れられ，放影研研究室に送られた．脂肪組織を除去した後，標本を小片に細切し，15％胎牛血清及び

mycin, 100 μg/ml). After two weeks in a 5% CO_2-95% air-regulated incubator at 37°C, fibroblast cells had attached to the dish surface and surrounded each piece of minced tissue. Cells were trypsinized from the primary culture dishes and replated into stock 60 mm diameter dishes for amplification of cell numbers. Eighteen hours prior to irradiation, subconfluent stock cell cultures were trypsinized and plated into experimental dishes. The cell concentrations varied depending on the anticipated amount of cell killing from cell plating and x-irradiation so that, after a two-week incubation period, approximately 50 macroscopic colonies would appear in each dish. After overnight incubation (approximately 18 hours), medium was removed from each dish prior to x-irradiation at room temperature. Cells were irradiated using a Softex X-ray machine operated at 40 kVp, 5 mA, with 0.2 mm aluminum external filtration (786 cGy/min calculated dose rate). Fresh medium was added to each dish after irradiation. For both single and split dose exposures, cells were returned to the incubator immediately after each irradiation. One week after irradiation, cells were refed with fresh medium. Cells were allowed to multiply and form macroscopic colonies during the two-week incubation period. The experiment was terminated when cells were fixed in 10% formalin and stained with Giemsa. Plating efficiencies (PE)* varied from 19% to 63%.

Evaluation of reproductive survival

Survival of reproductive integrity of cells was measured by counting the number of colonies containing at least 50 cells. The PE was estimated by dividing the number of observed colonies appearing in the control dishes (nonirradiated) by the number of cells plated into those dishes. The surviving fraction (SF)* and statistical analysis of the resulting data have been described.[11] Briefly, the multitarget, single hit model was fit to the data by the method of maximum likelihood assuming the numbers of colonies formed followed Poisson probability distributions.

抗生物質(ペニシリン 100IU/ml 及びストレプトマイシン 100μg/ml)を含むαMEM培地を入れたFalconプラスチックペトリ皿上に押し付けた．5% CO_2，95%空気，37°Cに調整した恒温器で2週間置いたところ，線維芽細胞が皿の表面に付着し，各組織切片を取り囲んだ．初代培養皿の細胞をトリプシン処理した後，細胞数を増加させるため，直径60mmの繁殖用皿に再び播種した．照射の18時間前，定常期に達していない保存培養細胞をトリプシン処理し，実験皿に移した．2週間培養した後，各皿当たり約50個のコロニーを肉眼で観察できるように，細胞の播種とX線照射により予想される細胞死の量によって，細胞濃度を変化させた．一晩(約18時間)培養した後，各皿から培地を除去し，室温でX線照射した．0.2mmアルミニウム外部濾過を用い，40kVp，5mAでSoftex X線装置を用いて細胞を照射した(算定線量率 786cGy/分)．照射後，各皿に新鮮な培地を加えた．1回照射及び複数回照射のいずれの場合においても，各照射後直ちに細胞を恒温器に戻した．照射の1週間後，培地を新鮮なものと取り替えた．2週間の培養期間中に細胞は増殖し，肉眼で観察できるコロニーを形成した．細胞を10%フォルマリンに固定し，Giemsa染色を行うことにより実験を終了させた．プレーティング効率(PE)*は19%～63%であった．

増殖生存能力の評価

少なくとも50個の細胞をもつコロニーを数えることにより，細胞の増殖生存能力を測定した．対照用培養皿(非照射)で観察されたコロニー数を，それらの皿に播種した細胞数で割ることにより，PEを推定した．生存率(SF)*及び得られたデータの統計的解析については，既に報告されている.[11] 簡単に言えば，形成されたコロニー数がPoisson確率分布に従うと仮定し，最大尤度法によって，データに多重標的の単一ヒットモデルを当てはめた．

PE – plating efficiency determined by dividing the observed number of colonies from the control group at the termination of an experiment by the original number of cells plated into the dishes.

PE— プレーティング効率とは，実験終了時に対照群に観察されたコロニー数を，皿に播種した細胞数で割って得た値である．

SF – surviving fraction is the fraction of radiation-treated cells that have survived the treatment divided by the original number of cells after adjusting for the PE; D_q – quasi-threshold dose.

SF— 生存率とは，放射線処理後の細胞数をPE調整後のもとの細胞数で割って得た値である；Dq— 疑閾値線量．

RESULTS

The responses of six cell strains (SF84.27, SF84.24, SF84.25, SF84.14, SF84.21, and SF84.13) to single-dose radiation exposures are shown in Figure 1. In all six strains, there is a definite curvature to the semilog-plotted data at the low doses resulting in nonzero quasi-threhold (D_q) values* and n values greater than 1. Table 1 lists these estimated survival parameter values and the associated standard errors.

結　果

単一線量照射に対する細胞株6種(SF84.27, SF84.24, SF84.25, SF84.14, SF84.21, 及びSF84.13)の反応を図1に示す. 細胞株6種のすべてにおいて, 低線量における片対数表示データから明確な曲線が認められ, 疑閾値(Dq)*はゼロでなく, n値は1より大きかった. これらの推定生存パラメーター値及び関連する標準誤差を表1に示す.

FIGURE 1　X-RAY SURVIVAL OF CELLS FROM SIX STRAINS OF HUMAN SKIN FIBROBLAST GROWN IN PRIMARY CULTURE

図1　初代培養で増殖したヒト皮膚線維芽細胞の細胞株6種から得た細胞のX線生存率

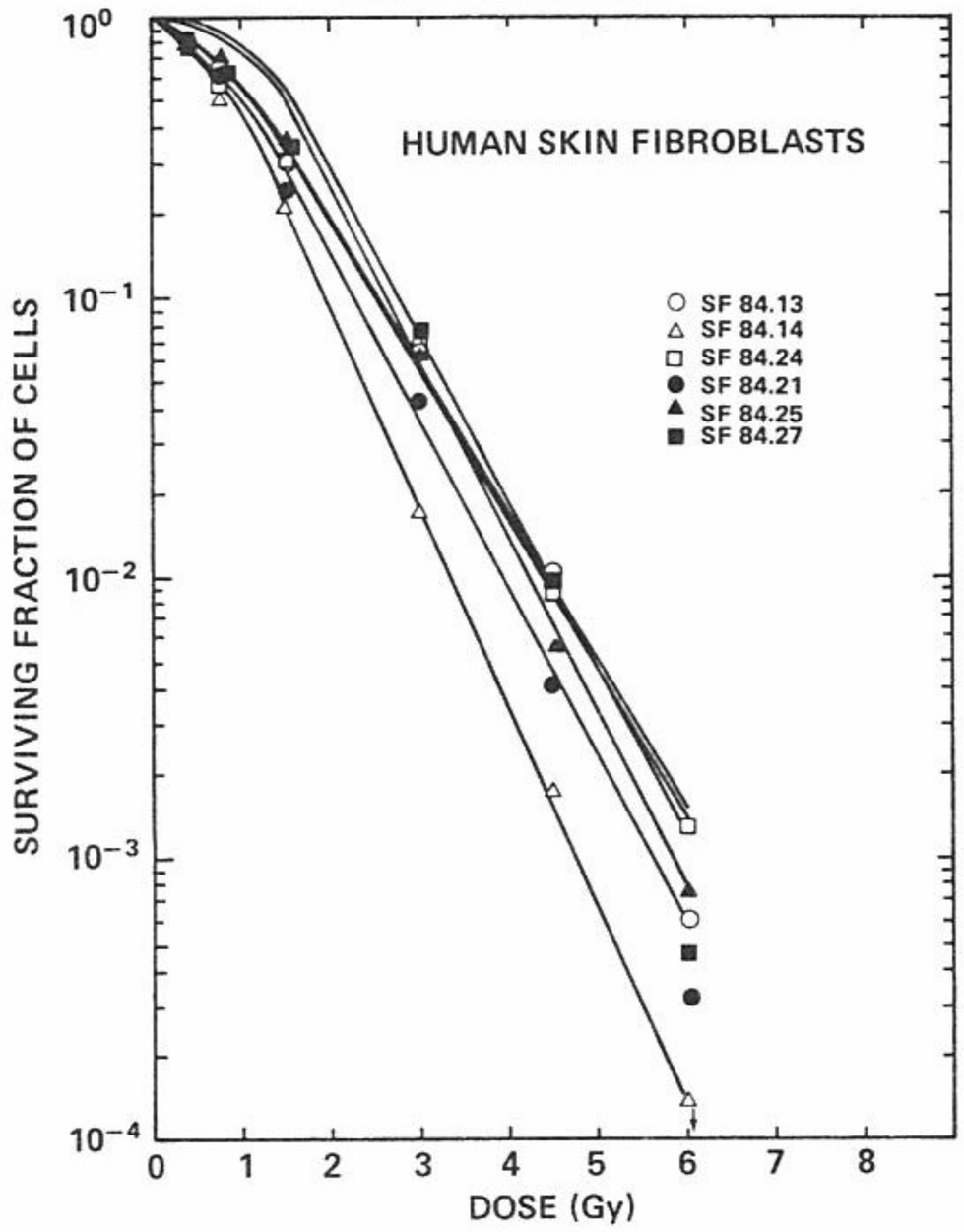


TABLE 1　DOSE-RESPONSE SURVIVAL PARAMETERS FOR SIX STRAINS OF HUMAN FIBROBLAST CELLS

表1　ヒト線維芽細胞の細胞株6種の線量反応生存パラメーター

Patient Number	D_0	D_q	n	PE
SF84.14	60.9 (2.4)	59.1 (8.7)	2.6 (0.5)	0.222
SF84.21	73.6 (2.5)	57.1 (10.5)	2.2 (0.4)	0.192
SF84.24	82.4 (2.1)	69.6 (9.1)	2.3 (0.3)	0.287
SF84.25	69.7 (1.8)	106.5 (9.2)	4.6 (0.8)	0.630
SF84.27	73.0 (2.5)	115.2 (13.1)	4.8 (1.1)	0.378
SF84.13	81.1 (2.3)	73.2 (9.8)	2.5 (0.3)	0.245

(SE of mean)　(平均の標準誤差)

Mean D_0 and D_q values given in cGy.　平均 D_0 値及び D_q 値の単位は cGy

To assess cellular repair of sublethal damage, cells were exposed to two equal doses of 152 cGy X rays separated by a time interval ranging from 0.5 to 5 hours. During the interval, cells were incubated at 37°C. As seen in the inserts of Figures 2a-f, cell survival increased for each cell strain as the time interval between fractions was increased. In most cases, survival had reached a plateau when the interval between dose fractions was at least three hours.

The dose-response curves for acute and two-fraction exposures are also shown in Figures 2a-f. For fractionation experiments, fibroblast cells were irradiated with 152 cGy X rays, incubated for four hours at 37° C, and then exposed to second graded X-ray doses from 38 to 453 cGy. The results of dose fractionation in general show steep negative initial slopes to the survival curves in response to the second dose. Survival, especially at the high doses, is significantly greater in cells exposed to fractionated X-ray doses than in singly exposed cells.

The amount of repair of sublethal damage for low and high dose fractions of X rays is shown in Figure 3. The recovery ratio, determined by dividing the surviving fractions of cells after exposure to a fractionated regime by the surviving fractions of singly exposed cells to that same total dose, has been previously described by Freeman et al.[9] Recovery ratios for the six cell strains are plotted as a function of dose. As the total dose increases above 250 cGy, the recovery ratios increase for all strains. However, the degree of recovery is highly dependent on the cell strain.

DISCUSSION

The radiosensitivities of six human skin fibroblast cell strains in primary culture were examined. Individual D_0 values varied from 61 to 83 cGy for the six strains. Although the low D_0 values indicate relatively high cellular radiosensitivity for the actively dividing cells, the survival curves were shouldered with n values ranging from 2.2 to 4.8. The relative biological effectiveness (RBE) of X rays from a spectrum of photons with a 40 keV maximum may account for the apparently high sensitivity of cells. Zeitz et al[12] have estimated the RBE of soft X rays (40 kVp) relative to ^{60}Co to be 1.4 when measured as a ratio of D_0 values.

細胞の亜致死性損傷の修復を評価するために，X線152cGy を，0.5～5時間の時間間隔で2回細胞に照射した．各照射の間は，細胞を37°C で培養した．図2a～f が示すとおり，照射間の時間間隔が増加するにつれて，各細胞株の細胞生存率は増加した．ほとんどの場合において，照射間の時間間隔が少なくとも3時間になったとき，生存率はプラトーに達した．

1回及び2回照射の線量反応曲線も，図2a～f に示す．複数回照射の実験においては，線維芽細胞に152cGy のX線を照射し，37°C で4時間培養した後，2回目には38-453cGy に分割したX線を照射した．概して，線量を分割した場合，生存曲線は第2回目の線量に対して，線量の少ない部分では急な負の傾きを示した．X線を1回照射した細胞より複数回照射した細胞の方が特に高線量の場合には生存率が有意に高かった．

低線量及び高線量のX線による亜致死性損傷の修復量を図3に示す．複数回の照射後の細胞生存率を，同じ合計線量を1回で照射した場合の細胞生存率で割ることにより決定する回復率については，以前 Freeman ら[9] が報告している．6種の細胞株の回復率を線量の関数として示した．合計線量が 250cGy より大きくなると，すべての細胞株において回復率が高くなる．しかし回復の程度は，細胞株の種類によって大きく左右される．

考　察

初代培養系の六つのヒト皮膚線維芽細胞株の放射線感受性を調査した．6種の細胞株の D_0 値はそれぞれ 61～83cGy であった．急速に分裂する細胞の D_0 値が低いということは細胞の放射線感受性が比較的高いことを示すが，生存曲線は2.2～4.8のn値をもち肩状を呈した．最高 40keV の光量子スペクトルからのX線の相対的生物学的効果比（RBE）によって，この細胞の感受性が明らかに高いことが説明できるかもしれない．Zeitz ら[12] は，^{60}Co に対する軟X線（40kVp）の RBE を D_0 値の比率として測定した場合，1.4であると推定している．

FIGURE 2 REPAIR OF RADIATION-INDUCED SUBLETHAL DAMAGE IN SIX STRAINS (a-f) OF HUMAN SKIN FIBROBLASTS AFTER SINGLE OR TWO FRACTION DOSES OF X RAYS

図２　１回又は２回Ｘ線照射後のヒト皮膚線維芽細胞の細胞株６種（a-f）における放射線誘発亜致死性損傷の修復

Figure 2a

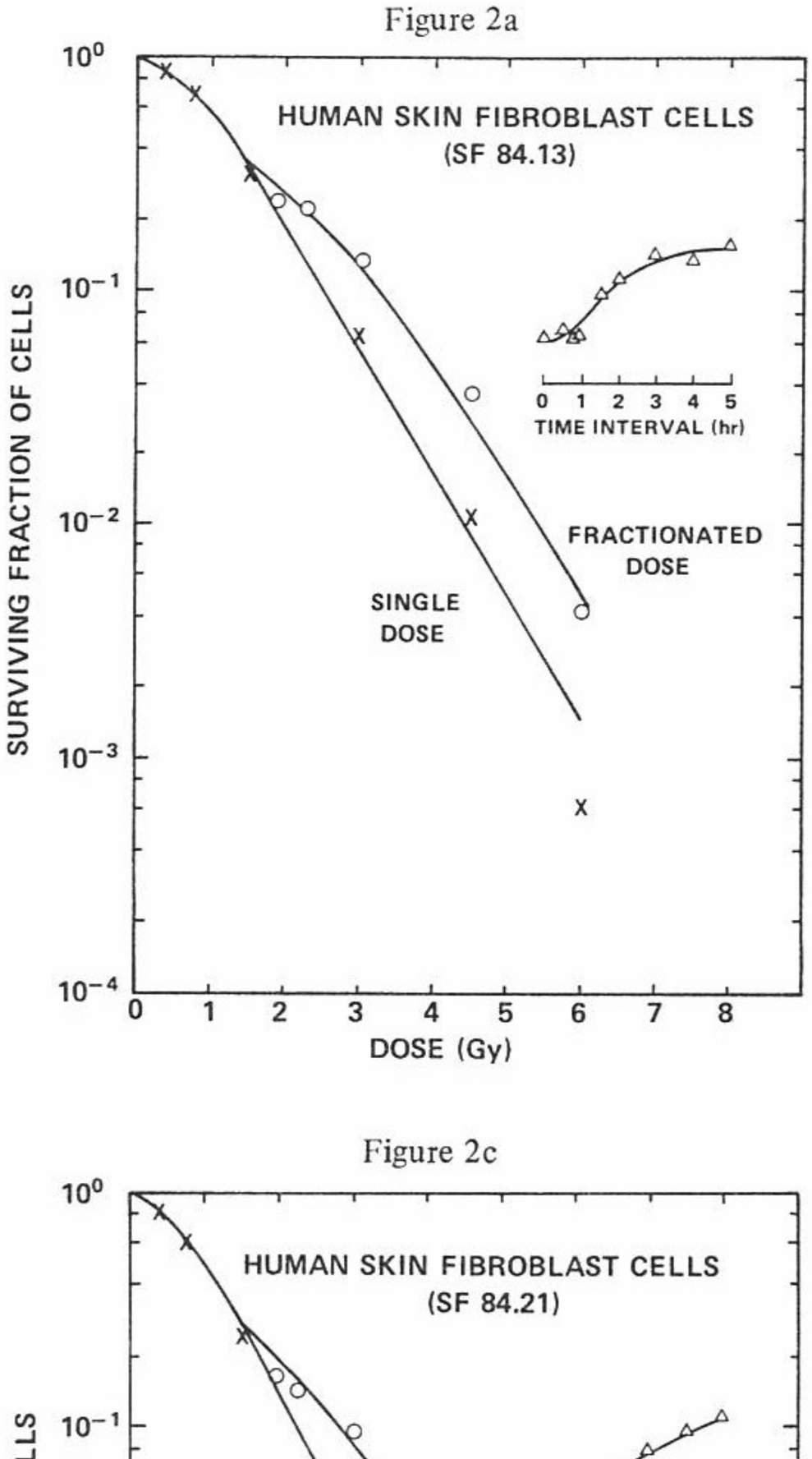


Figure 2b

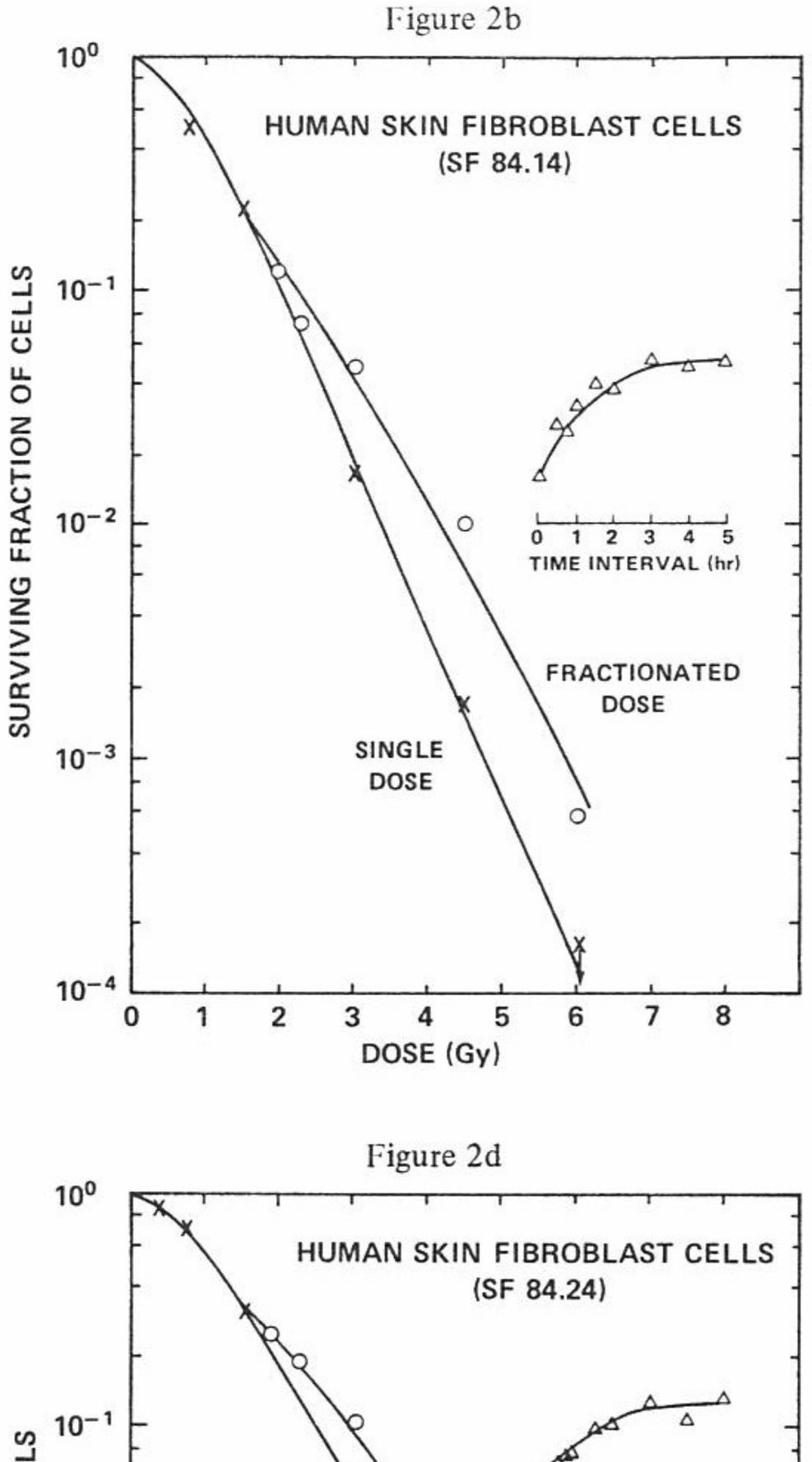


Figure 2c

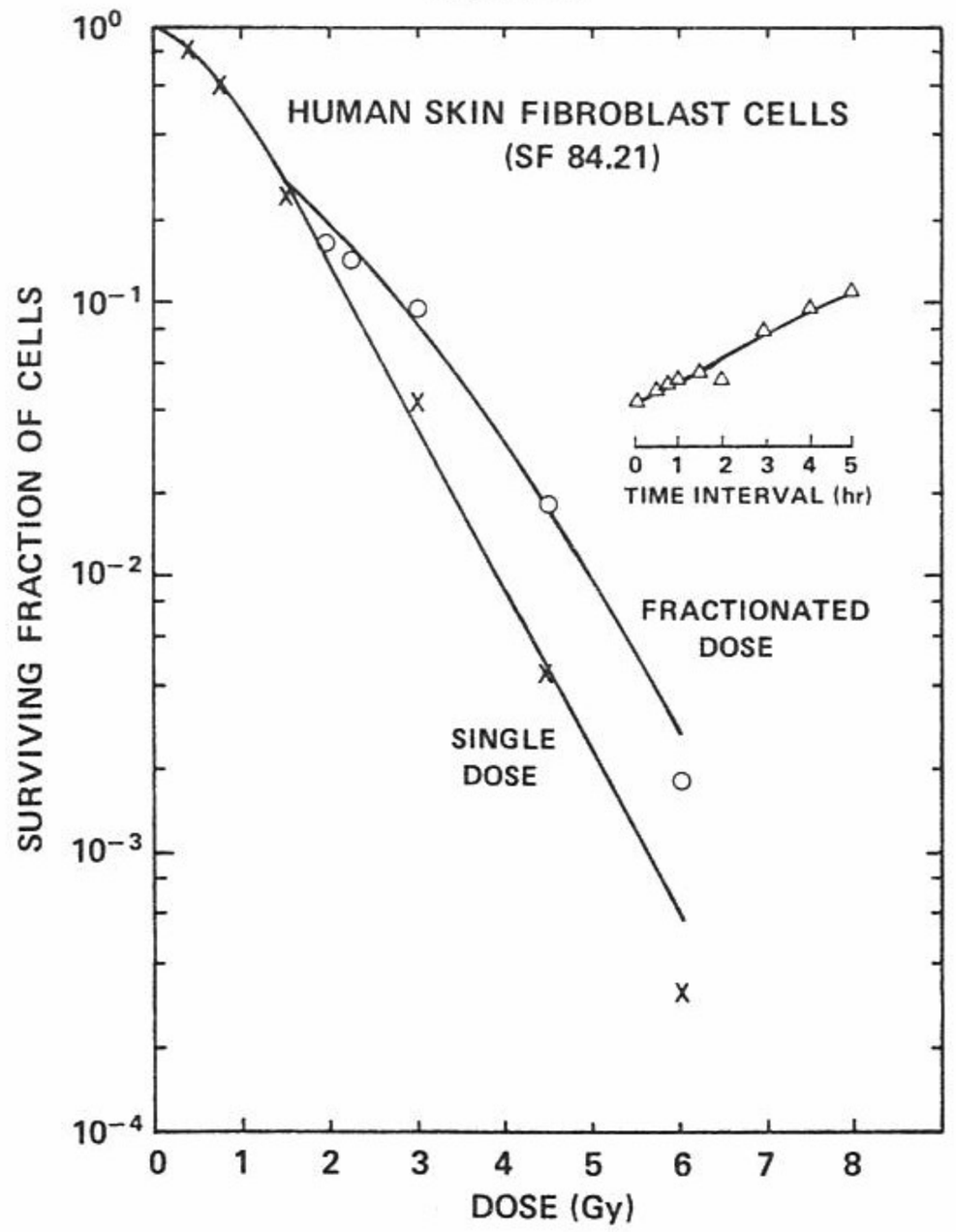


Figure 2d

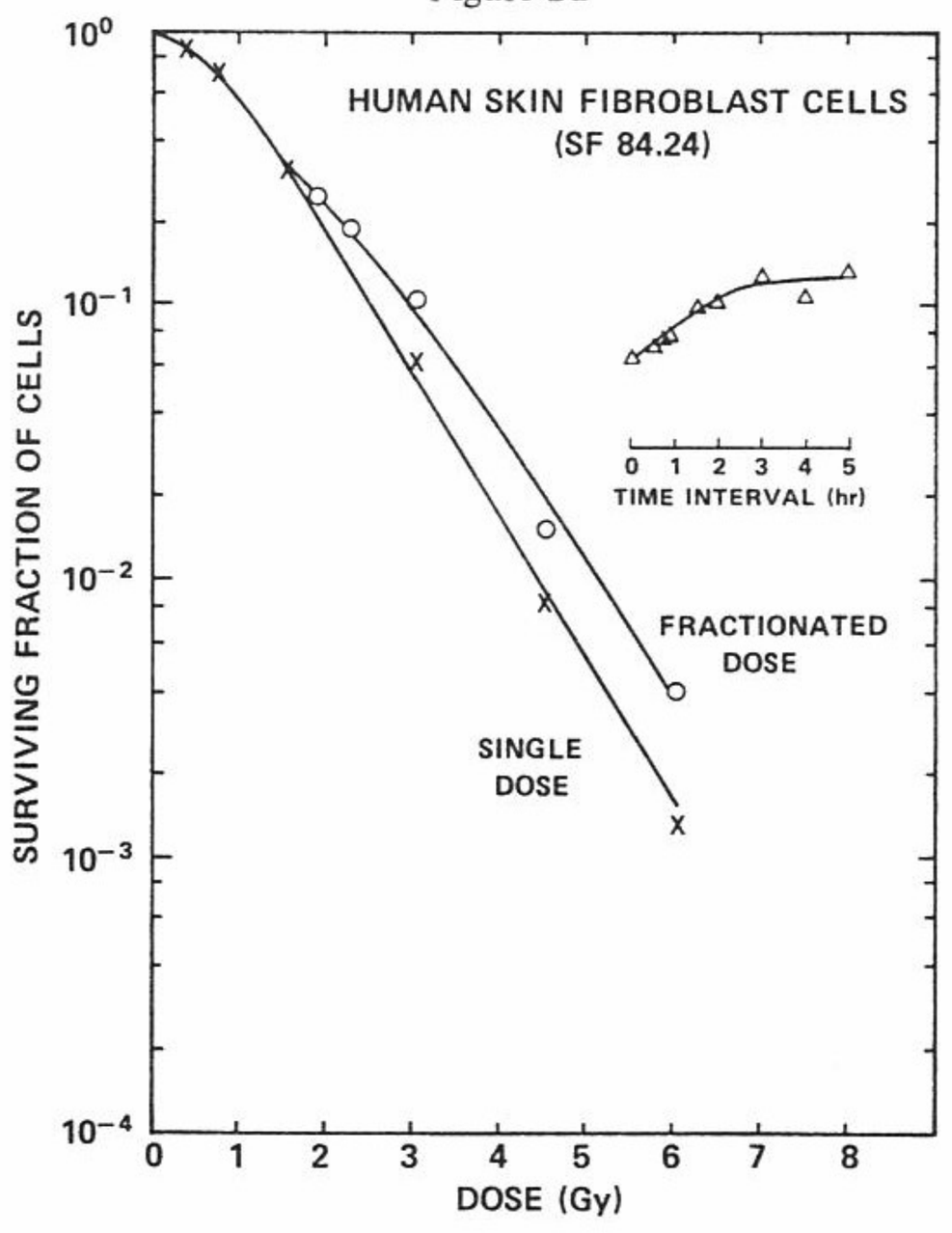


Figure 2e

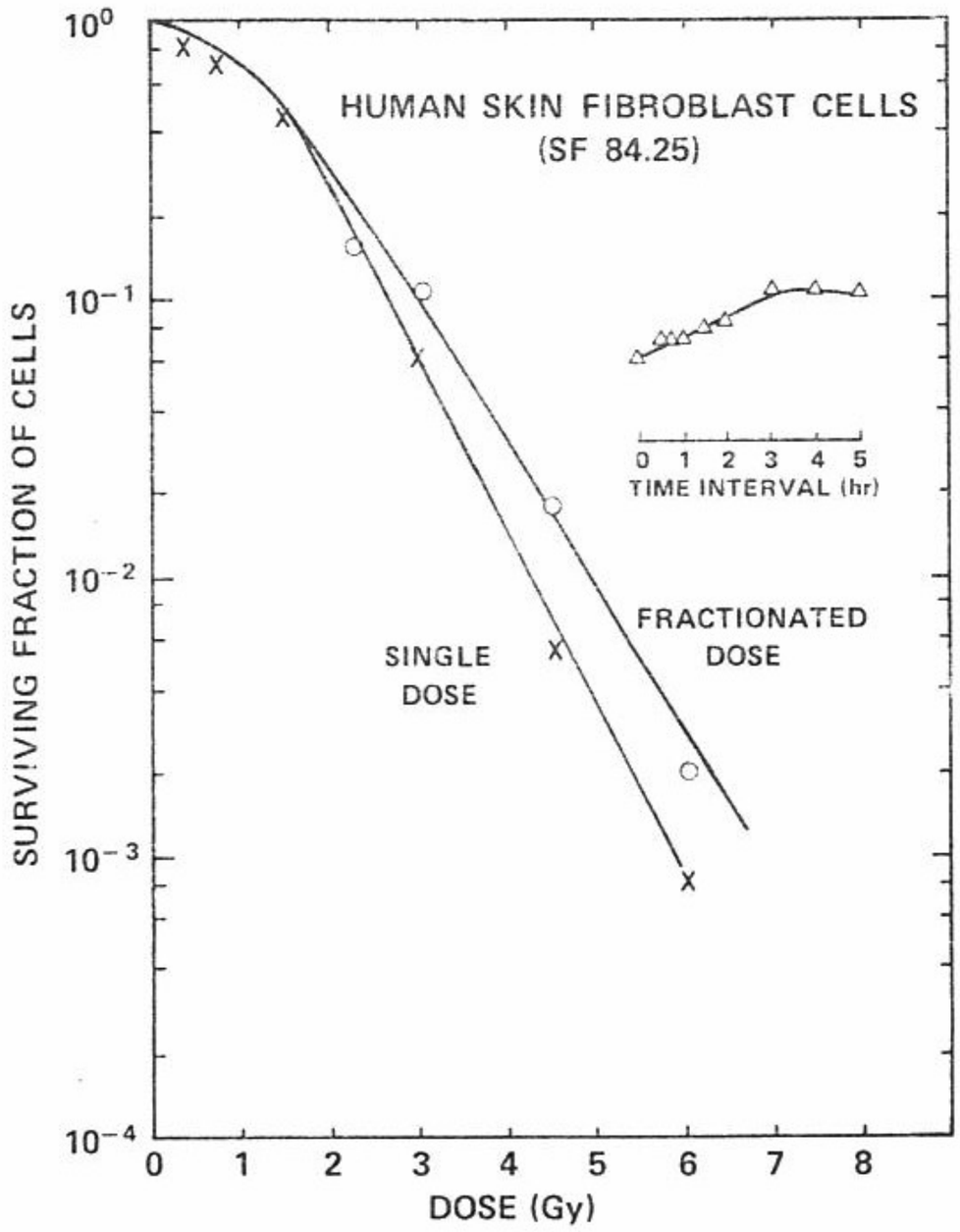
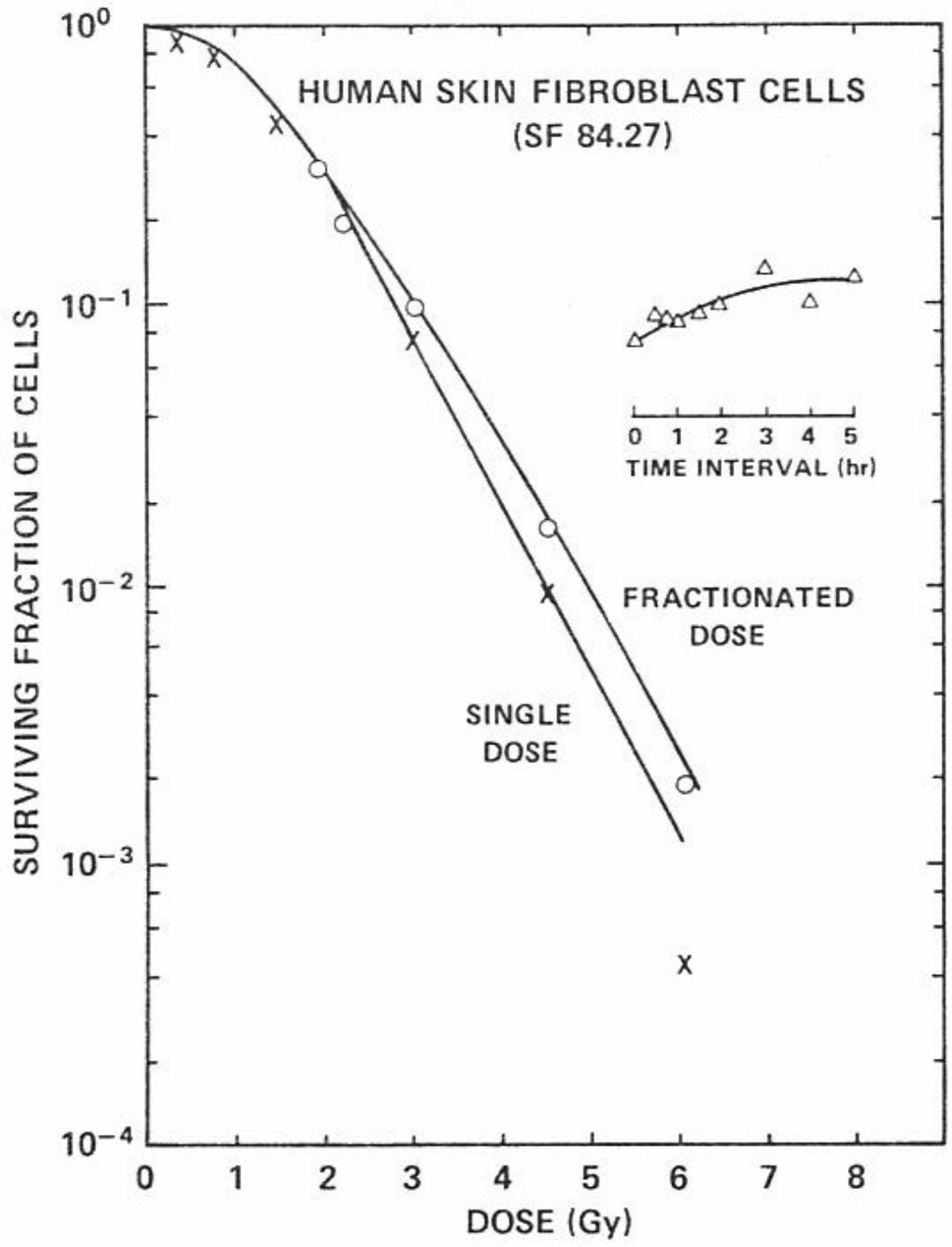


Figure 2f



Irradiation procedures and statistical analyses are described in the text.
照射手順及び統計的解析に関しては本文を参照.

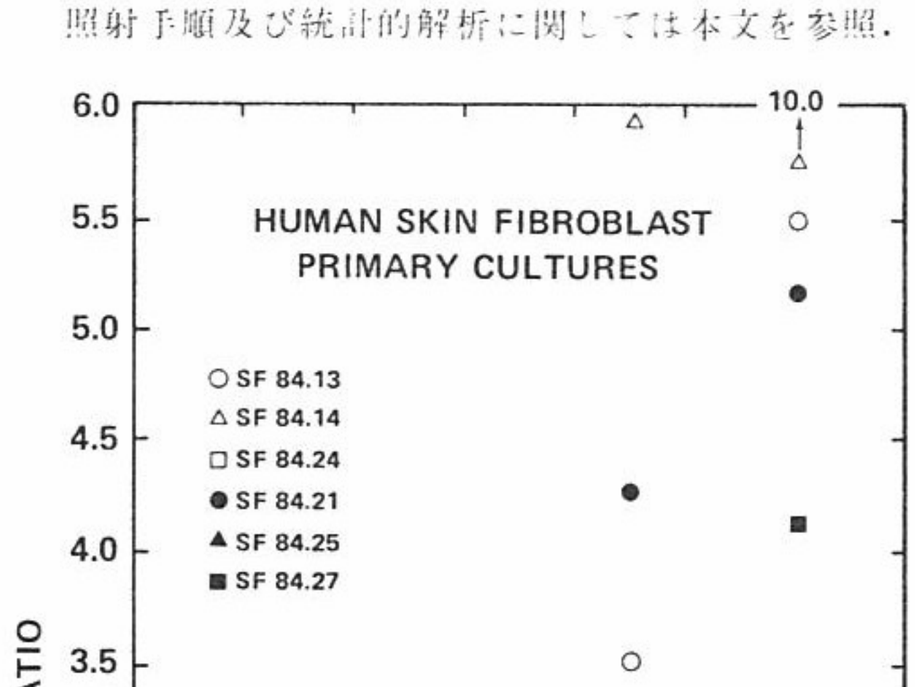


FIGURE 3 RECOVERY RATIOS OF CELLS SURVIVING SINGLE AND TWO FRACTION EXPOSURES OF X RAYS. RECOVERY RATIOS AS A FUNCTION OF DOSE ARE SHOWN FOR THE SIX STRAINS OF HUMAN FIBROBLASTS

図3 1回又は2回X線照射後の生存細胞の回復率. ヒト線維芽細胞の細胞株6種について, 線量の関数としての回復率を示す

Dose fractionation experiments confirm the ability of cells to recover from sublethal damage. In all cell strains, maximal cell survival was observed when the time interval between two 152 cGy fractions was more than three hours. A single exposure of 152 cGy to cells results in approximately 70% cell killing and was chosen because it is about two times the D_0 for cell killing. In oncogenic transformation studies performed in vitro,[13,14] exposure of cells to approximately twice their D_0 values is often sufficient to induce maximal frequencies of cellular transformations.

When cells were exposed to 152 cGy X rays, incubated at 37° C for four hours to allow for recovery from the first dose before being given second graded doses, the subsequent fractionated dose-survival curve was distinctively different from the single dose exposure curve. Therefore, recovery had occurred during the four-hour interval. In addition, as the second graded dose increased, the relative degree of recovery increased as shown by the difference in the two slopes. The recovery ratio increase as a function of dose is clearly illustrated in Figure 3 where it is apparent that the enhancement of survival after dose splitting is measureable only for second graded doses above 250 cGy and is greatest at high doses.

While some investigators have reported that cultured diploid human fibroblasts are unshouldered and exponential throughout the range of doses,[1-3] our results are in agreement with those studies that report recovery from sublethal radiation damage by human fibroblast cells.[5-9] The small shoulders seen in these radiation dose-survival curves may not accurately reflect the repair capacity of cells in vivo. For example, Gould et al[15] and Mulcahy et al[16] have recently described an in situ repair process in epithelial cells of rat hepatocytes and thyroid. They were able to enhance survival of cells (enlarged shoulder) by simply leaving cells undisturbed in situ for at least four hours after irradiation before assaying them by the transplantation technique.[15] It is likely that the ability of human fibroblast cells to repair radiation damage is present but difficult to measure by the usual cell survival techniques.

線量分割照射実験により，細胞が亜致死性損傷から回復する能力をもつことが確認された．すべての細胞株において，152cGy の2回の照射間の時間間隔が3時間以上のときに，細胞の生存率が最高であった．細胞に 152cGy を1回照射すると細胞致死率が約70%になり，D_0 値の細胞死の約2倍であるので，この線量を選択した．試験管内で行った腫瘍性転換に関する研究[13,14]では，多くの場合細胞転換の頻度を最大にするためには，細胞をその D_0 値の約2倍の線量に被曝させれば十分である．

細胞にX線 152cGy を照射し，第1回目の照射からの回復のため 37°C で4時間培養した後，第2回目の照射を行った場合，それ以降の線量一生存曲線は，単一線量被曝曲線とは著しく異なっている．したがって，その4時間の間に回復が行われている．更に，二つの傾きの差異に示されるとおり，第2回目の線量の増加に伴い回復の相対度が増加した．線量の関数としての回復率増加が図3に明白に示されているが，線量分割後の生存率の増加は，第2回目の線量が 250cGy を上回るときにのみ明らかであり，高線量において最大である．

幾人かの研究者は，培養ヒト二倍体線維芽細胞はいずれの線量においても肩状を呈さず，指数関数的であると報告しているが，[1-3] 我々が得た結果は，ヒト線維芽細胞が亜致死性損傷から回復するという報告[5-9]と一致する．これらの放射線量一生存曲線に認められる小さな肩は，生体内の細胞の修復能力を正確には反映しないかもしれない．例えば，Gould ら[15]及び Mulcahy ら[16]は最近，ラットの肝細胞及び甲状腺の上皮細胞における in situ の修復過程について報告している．彼らは，照射後少なくとも4時間 in situ に細胞を放置するだけで，その後移植により測定を行うと細胞の生存率が高くなること（肩の拡大）に成功した．[15] ヒト線維芽細胞は放射線損傷を修復する能力をもっているが，それを通常の細胞生存技法により測定することは困難であると考えられる．

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