# Different Doses of Radiation on Agar Colony Forming Development in C6 Glioma Cells: Assessment by Thymidine Labeling Index, and Bromodeoxyuridine Labeling Index

## *C6 GLİOMA HÜCRELERİNDE DEĞİŞİK RADYASYON DOZLARINDA KOLONİ OLUŞTURMA ETKİSİ: TİMİDİN İŞARETLEME İNDEKSİ VE BROMODEOKSİÜRİDİN İŞARETLEME İNDEKSİ İLE DEĞERLENDİRME*

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## Abstract

**Objective:** Gliomas are relatively frequent in adults, and are among the most malignant primary brain tumors. Glioblastoma multiforme, like many other tumors that exhibit radiation *sensitivity in vitro*, seems to be very resistant to radiation *in vivo*, thus suggesting that irradiation may not be a rate-limiting factor for malignant glioma tumor growth. In this study, we aimed to determine the optimal dose of radiation in C6 glioma colony forming assay, which is a valuable tool for antitumor treatment screening.

**Material and Methods:** $10^5$ cell/lamella colony forming cells were radiated with 200 cGy, 400 cGy, 800 cGy and 1600 cGy for 10 minutes. Radiosensitivity was measured systematically 24, 48, 72 and 96 hours after the radiation by three methods: soft-agar bilayer assay, thymidinE incorporation, and bromodeoxyuridine (BrdU) incorporation.

**Results:** The soft-agar bilayer assay, which assessed the colony-forming units, showed that the number of colonies in the control group (609, 3 ± 86.8) were decreased after 200 cGy (8.3 ± 3.6) and 400 cGy (7.2 ± 4.3). No colony was detected in 800 cGy and 1600 cGy irradiated cells [3H] Thymidine incorporation was more prominent with higher doses of radiation. BrdU incorporation revealed that even at low doses (200 cGy) of radiation there was a significant decrease of cell proliferation. On higher doses like 1600 cGy it was more prominent.

**Conclusion:** Cell survival, doubling time, and cell phases are parameters of growth kinetics, and the results suggest that C6 glioma cells are radiosensitive and are virtually affected by all radiation doses in our experiment even 200 cGy at 24 hours. Besides, colony forming assay with thymidine labeling index, and BrdU labeling index may be used as new methods for determining radiotherapy doses in clinical applications.

**Key Words:** Dose fractionation; glioma

## Özet

**Amaç:** Gliomalar erişkinde oldukça sık görülen, kötü gidişli primer beyin tümörlerinin büyük bölümünü oluştururlar. Pek çok tümör gibi glioblastoma multiforme de in vitro radyasyona duyarlıdır, ancak in vivo radyasyon direnci gösterir. Bu sebeple malign glioma tümör gelişiminde, radyasyon tedavisi sınırlayıcı olmayabilir. Bu çalışmada, anti tümör tedavi taramasında çok uygun bir teknik olabilen "colony forming assay" yöntemi ile C6 glioma hücre hattında en uygun radyasyon dozunun belirlenmesi amaçlandı.

**Gereç ve Yöntemler:** $10^5$ hücre/lamel içeren "colony forming" hücreleri 10'ar dk. boyunca 200 cGy, 400 cGy, 800 cGy ve 1600 cGy radyasyona maruz bırakıldı. Radyasyon alan hücrelerdeki radyosensitivite sistematik olarak 24, 48, 72 ve 96 saatlerde "soft-agar bilayer assay" timidin (TLI) ve BrdU (BLI) işaretleme indeksleriyle değerlendirildi. "Soft-agar bilayer assay" oluşan koloni sayılarını belirlemekteydi ve bu sayı kontrol grubunda (609.3 ± 86.8) iken 200 cGy (8.3 ± 3.6) ve 400 cGy (7.2 ± 4.3)'de belirgin olarak azalmıştı. 800 cGy ve 1600 cGy sonrası koloniye rastlanamadı.

**Bulgular:** TLI yüksek dozda, BLI ise düşük dozda (200 cGy) radyasyonun değerlendirilmesinde daha güvenilir bir tekniktir. Bu çalışmada düşük doz (200 cGy) radyasyon sonrasında bile hücre proliferasyonunda belirgin bir azalma olmuştur. Bu 1600 cGy yüksek dozda çok daha belirgindir.

**Sonuç:** Hücre yaşam süreleri, ikilenme zamanları ve hücre fazları büyüme kinetiklerini gösterir. C6 glioma hücreleri radyosensitiftir ve 200 cGY radyasyon dozunda ve 24 saatte bile açıkça etkilenirler. Bununla beraber TLI ve BLI klinik uygulamalarda radyoterapi dozunun belirlenmesinde yeni bir metod olarak kullanılabilir.

**Anahtar Kelimeler:** Radyoterapi doz ayarlama; glioma

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Malignant glioma is the most common primary tumor of the brain in adults and has a very poor prognosis. Despite advances in surgery, chemotherapy, radiotherapy and imaging techniques no clear trend toward

improvement in outcome was achieved for malignant glioma.[1-3] Local recurrence occurs in 90% of patients and their survival is extremely low. New molecular targets and new therapeutic modalities are essential for further treatments.[4] Currently, modern and advanced radiotherapy systems are effective as local treatments of malignant tumors.

While planning for radiotherapy, not only the parameters such as tumor size, tumor invasion and lymphatic involvement but also tumor cell characteristics should be taken into consideration. There are lots of effective methods to define the cell characteristics of tumors. Creating colonies in agar is a method which is called double layer agar colony forming assay.[5] With double layer agar colony forming assay, the effects of different antineoplastic drugs and radiotherapy on primer tumor colonies can be comparatively studied. Thymidine labeling index (TLI) and BrdU, have also been used successfully to define tumor cell characteristics.[6]

Recent studies show that irradiation of C6 glioma cells, decreased their proliferation in vitro in a dose-dependent manner.[7] In this study, we treated C6 glioma cells of rats with different doses of radiation. Our aim was to determine the optimal dose of radiation by comparing colony forming assay with TLI and BrdU index. Dose-dependent radiotherapy represents a new therapeutic approach in clinical applications and we aimed to examine the proper dose and method for clinical applications in patients.

## Material and Methods

### Cell Culture

C6 glioma cells were obtained from American Culture Collection. This tumor cell line is maintained in culture as adherent cells and cultured in DMEM (Sigma Chemical Co., St Louis, Missouri) plus 10% heat inactivated fetal calf serum (FCS) (Sigma Chemical Co., St Louis, Missouri), supplemented with 1% L glutamin (Sigma Chemical Co., St Louis Missouri), 1% nonessential amino acids (Sigma Chemical Co., St Louis, Missouri), 10.000 units/mL penicillin (Sigma Chemical Co., St Louis, Missouri), and 10 mg/mL streptomycin (Sigma Chemical Co., St Louis, Missouri). Cell line was grown in a humidified atmosphere at 37°C in 5% $CO_2$. When the tumor cell lines were used as target cells, they were treated with tyripsin-EDTA (Sigma Chemical Co., St Louis, Missouri), washed, and resuspended in complete medium. Dimethylsulfoxide (DMSO) and trypan blue dyes were purchased from Sigma (Sigma Chemical Co., St Louis, Missouri). Stock solutions of the reagents were prepared in PBS, medium or DMSO as appropriate. A total of 1.500.000 cells were used (500.000 cells for TLI, 500.000 cells for BrdU and 500.000 cells for agar colony development). The cells were seeded in sterile 25 $cm^2$ culture flasks with 5 mL medium as described. This study was performed in İstanbul University Medical Faculty Department of Histology and Embryology and passages were controlled with invert microscope at least twice a week.

### Agar colony development

After the cultures became semiconfluent, flasks were treated with 200, 400, 800 and 1600 cGy radiation. Radiation was applied for 10 minutes for each dose in flasks. After about an hour, the cells were treated with tyripsin and were counted. Then, 1000 cells were added on the upper layer of each well of the culture plates. The plates were incubated for 7 days. After this period, the colonies were evaluated and counted.

Three wells were inoculated for each radiation dose.

The group of cells containing at least 30 cells was accepted as a colony.

The mean values and standard deviations of counted cells were calculated.

### TLI of Tumor Cells

After the cultures became semiconfluent the cells in one flask were treated with tyripsin (0.25%) and the resulting cell pellet was inoculated on coverslips placed in sterilized 24 well-culture plates with a final concentration of 1 x $10^5$ cell/coverslip in 2 mL of medium for each experiment. The attachment of cells on the coverslips were controlled by invert microscope and the cells were radiated for 10 minutes with 200 cGy, 400

cGy, 800 cGy and 1600 cGy for each plate. Systematically 24, 48, 72 and 96 hours after the radiation, cells were treated with 1 mCi/mL 3H TdR for 30 minutes. Then cells were fixed with Carnoy fixative and autoradiography was performed. The percentage of labeled cells over at least 1000 cells from each coverslip was calculated. For each group 3 coverslips were used. The mean values and standard deviations of the results were evaluated.

**BrdU Labeling Index of Tumor Cells**

Cells in the S-phase of the cell cycle (thus synthesizing DNA) can be labeled by BrdU incorporation into the DNA strand replacing thymidine and can be subsequently detected by immunocytochemical means. C6 glioma cells were incubated with 0.3% $H_2O_2$ for 10-15 minutes to inhibit endogenous peroxidase activity. Then, sections were treated with trypsin at 37°C for 30 min and rinsed with water. After buffered phosphate saline incubation for 5 minutes at 37°C, sections were treated with 2 N HCl for 30 minutes at 37°C to denature DNA. Sections were incubated in 0.1 M Borax for 10 minutes and then in PBS for 5 minutes at room temperature. Incubation with Ultra V Block for 5 min, primary antibody (Sigma Chemical Co., St Louis, Missouri 1:200 dilution) for 1 hour, secondary antibody (biotinylated goat anti-mouse) for 10 minutes, streptavidin peroxidase for 20 minutes and peroxidase-compatible chromogen for 20 minutes in incubation box at room temperature were performed by using Ultravision Large Volume Detection System Anti-Mouse HRP Kit (Lab Vision, California, USA) and AEC Substrate System Kit (Lab Vision, California, USA). Sections were rinsed with water and counterstained in Mayer's Hematoxylin for 3 minutes. BrdU labeled cells had red stained nuclei.

Statistical Analysis

The obtained data were compared between and within groups using ANOVA with Bonferroni test. Differences were considered to be significant at p values less than 0.05. All results are expressed as mean SEM calculated from at least 3 independent experiments. All statistical analyses were performed with GraphPads Instat 3.05 software (GraphPad Software Inc., San Diego, CA, USA).

## Results

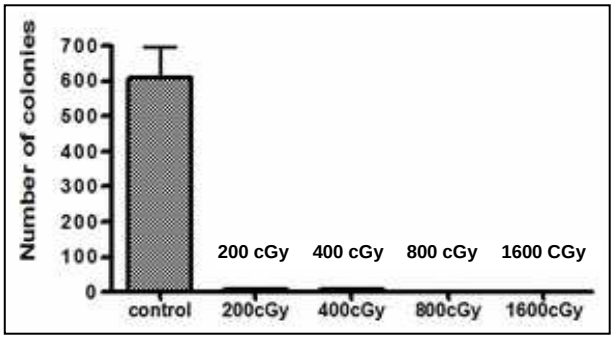
### Radiotherapy Decreased the Number of Colonies

Number of colonies in the control group (609.3 ± 86.8) were decreased after 200 cGy (8.3 ± 3.6) and 400 cGy (7.2 ± 4.3) irradiation. No colony was detected in the 800 cGy and 1600 cGy irradiated cells. Data obtained from the controls were significantly different than those of the 200 cGy ($p< 0.0001$) and 400 cGy ($p< 0.0001$) irradiated cells (Table 1, Graphic 1). However, ANOVA showed that there was no difference between the 200 cGy and 400 cGy groups ($p> 0.05$). In addition to the reduction in the number of colonies, smaller colonies were determined in the radiotherapy groups.

**Table 1.** Number of colonies.

| | Mean | SEM |
|---|---|---|
| Control | 609.3 | 86.8 |
| 200 cGy | 8.3 | 3.6 |
| 400 cGy | 7.2 | 4.3 |
| 800 cGy | 0.0 | 0.0 |
| 1600 cGy | 0.0 | 0.0 |

SEM: Standard error of the mean.




**Graphic 1.** Number of colonies in the control group was significantly lower than those in the 200 cGy and 400 cGy cells. There was no colony in the 800 cGy and 1600 cGy cells. Differences between the controls and the 200 cGy ($p< 0.0001$) and 400 cGy ($p< 0.0001$) groups were statistically significant.

**Table 2.** Evaluation of TLI after irradiation.

| | Control | | 200 cGy | | 400 cGy | | 800 cGy | | 1600 cGy | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 24 hours | 39.50 | 10.70 | 13.40 | 12.80 | 41.99 | 10.36 | 33.25 | 11.37 | 31.25 | 10.14 |
| 48 hours | 42.40 | 11.20 | 41.20 | 12.40 | 33.57 | 12.73 | 29.22 | 12.45 | 34.50 | 15.62 |
| 72 hours | 48.60 | 13.38 | 40.78 | 14.11 | 27.70 | 14.01 | 22.70 | 11.23 | 27.70 | 14.01 |
| 96 hours | 13.51 | 3.34 | 17.14 | 8.76 | 20.98 | 8.72 | 16.25 | 9.10 | 1.43 | 1.22 |

TLI: Thymidine labeling index,
SEM: Standart error of men.

**Table 3.** Evaluation of BrdU after irradiation.

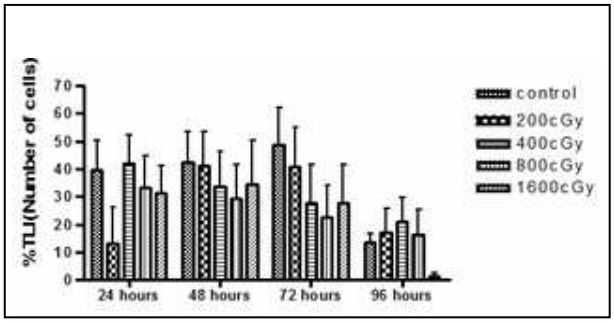
| | Control | | 200 cGy | | 400 cGy | | 800 cGy | | 1600 cGy | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 24 hours | 4.47 | 3.30 | 1.52 | 3.60 | 1.49 | 1.38 | 1.10 | 2.39 | 0.67 | 2.01 |
| 48 hours | 14.90 | 5.13 | 7.06 | 5.11 | 2.01 | 2.50 | 2.22 | 2.04 | 4.32 | 4.35 |
| 72 hours | 18.63 | 6.96 | 1.06 | 3.15 | 0.90 | 2.12 | 3.06 | 3.89 | 4.44 | 2.66 |
| 96 hours | 10.70 | 5.55 | 2.98 | 4.79 | 2.47 | 2.87 | 2.50 | 2.16 | 1.81 | 2.99 |

BrdU: Bromodeoxyuridine,
SEM: Standart error of men.

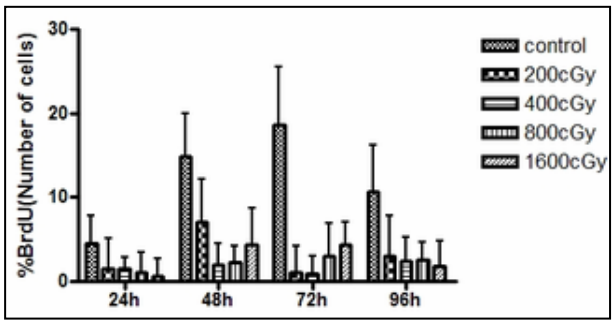
### Irradiated C6 Glioma Cells Decreased Percentage of S Phase Fraction of Cells

**TLI:** Particularly 200 cGy radiation affected C6 glioma cells at 24 hours of observation for TLI ($p < 0.0001$). Higher doses of radiotherapy did not affect the cells significantly ($p > 0.005$) at 24 hours. However, further observations clearly revealed that 400, 800 and 1600 cGy radiotherapies influenced glioma cells evidently at 48, 72 and 96 hours (Table 2, Graphic 2). Overall our results clearly show that irradiation induces a significant reduction in the percentage of S phase fraction of cells. This effect is more prominent in higher doses of radiation.

**BrdU labeling index:** Results of BrdU labeling index showed that even at low doses (200 cGy) of radiation cell proliferation significantly decreased. On higher doses like 1600 cGy the effect was more prominent. At 24 hours there was a significant difference between the control group




**Graphic 2.** The most prominent effect at 24 hours of observation for TLI was with 200 cGy radiation of C6 glioma cells ($p < 0.0001$). Higher doses of radiotherapy did not affect the cells significantly ($p > 0.005$) at 24 hours. However, further observations clearly revealed that 400, 800 and 1600 cGy irradiation influenced glioma cells evidently at 48.72 and 96 hours.




**Graphic 3.** In all radiotherapy groups, even the 200 cGy, BrdU stained cell numbers were significantly decreased ($p < 0.0001$) and 400, 800 and 1600 cGy radiotherapies influenced glioma cells evidently at 48, 72 and 96 hours.

and 200cGy radiation group. However, there was statistically no difference between higher doses and 200 cGy at 24 hours of observation. Although at 48 hours of observation there was a statistically significant difference between 200 cGy and 400 cGy, no difference was detected between higher doses and 400 cGy (Table 3, Graphic 3).

### Changes on Cell Morphology after Radiotherapy

Thymidine and BrdU positive cells were detected with immunohistochemical and monoclonal staining techniques (Figure 1, 2). Control cell shapes reflecting regular C6 glioma cell morphology were used. In the control group, the number of positive stained cells was higher than in the irradiated cells. Highly proliferative C6 glioma cells were significantly decreased after irradiation even low dose radiation with thymidine and BrdU incorporation (Figure 3, 4). A decrease in the cell number as well as poor staining of DNA was observed in radiotherapy groups. Morphological changes indicated that radiotherapy affected cells particularly starting from low doses.

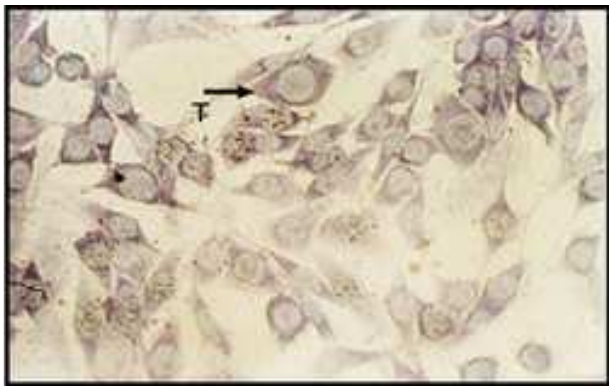



**Figure 1.** C6 glioma cells with thymidine labeling without radiotherapy. Arrow shows positive staining cell with dark cytoplasm (x 400).

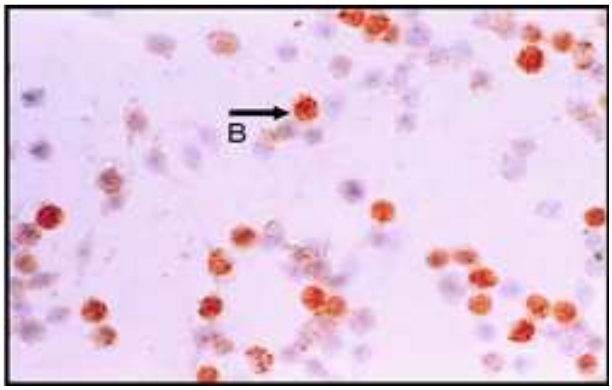



**Figure 2.** BrdU positive cells were detected in the control group. Arrow shows BrdU positive cell (x 400).

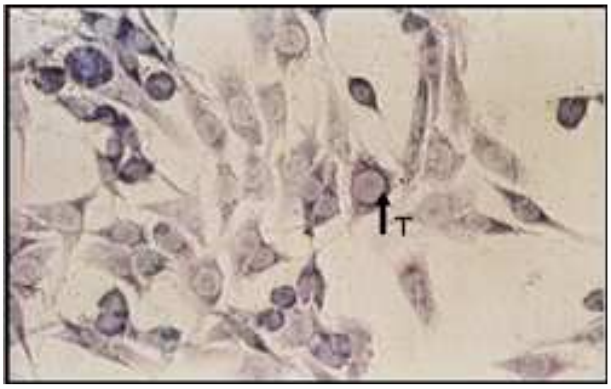



**Figure 3.** After radiotherapy, numbers of labeling cells with Thymidine are significantly decreased. Arrow shows positive staining cell (x 400).

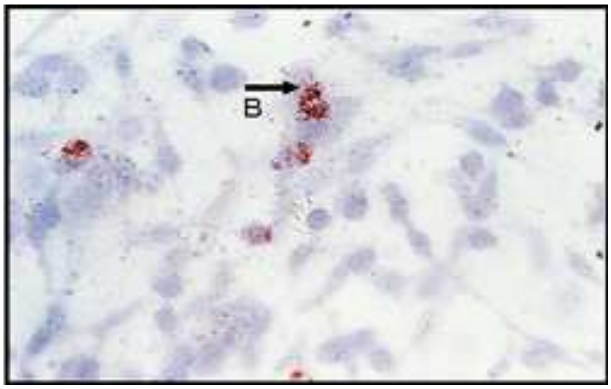



**Figure 4.** Highly proliferative C6 glioma cells were significantly decreased after radiotherapy even at low dose. Arrow shows positive BrdU incorporation (x 400).

## Discussion

The aim of this project was to realize a new method-colony forming efficiency with TLI and BrdU labeling index-to determine radiotherapy doses. TLI was one of the first methods utilized to evaluate the proliferative activity of cancer cells. The number of tumor cells undergoing DNA synthesis can be measured using in vivo or in vitro assays for 3H-thymidine uptake, which can be visualized by autoradiography.[8] The TLI expresses

the ratio between thymidine-labeled cells and the total number of tumor cells and was shown to be reliable and stable over time.[9] Labeled pyrimidine bases other than (3H) thymidine, such as the halogenated analogue BrdU, have also been used successfully.[10,11] Of note is that incorporated BrdU may be revealed by immunohistochemistry (IHC). The technique, however, has limitations that have hampered its acceptance as a standard method. In fact, fresh tumor tissue or cultured cells are needed and a complex and time-consuming radioactive assay or in vivo administration of labeled substances is required.[12]

Our main goal was to combine colony forming assay with both TLI and BrdU indexes. The modest benefit seen was thought to be related to the heterogeneous distribution of the halogenated pyrimidines in tumors and the low thymidine replacement achieved in individual tumor cells. BrdU is a potent radiosensitizer when incorporated into the DNA of target cells; however, the degree of radiosensitization depends on the extent of thymidine replacement by the analog and on the number of cells labeled. The failure of proliferating cells to take up thymidine analogs such as BrdU is attributable to the low availability of the drug to the tumor cell or to the analog being diluted to below useful levels by endogenous nucleotide precursors. The latter results from the activity of enzymes in the *de novo* synthesis pathways and/or the enzymes in the alternative pathways for pyrimidine or purine biosynthesis which are so-called salvage pathways. A marked rise in the activities of enzymes in both categories has been observed in cancer cells in logarithmic growth and in hepatomas of different growth rates.[13]

Cancers are spreading rapidly among humans and new diagnosis and treatment modalities are essential for the cure of this disease. The optimal treatment for recurrent high-grade glioma remains undefined despite advances in surgery, chemotherapy and radiotherapy.[14-17] Attempts to improve the response of patients to radiotherapy have involved a number of stratagems. Malignant glioma tumors exhibit various behaviors in response to radiotherapy. This is due to the diversity of radiation sensitivity in laboratory conditions and human body.[7] C6 glioma cells are quite resistant to irradiation in vitro, even at high doses of X-rays.[1] This significant data was the inspiration of this project. Other tumors, even glioblastoma multiforme cell lines that exhibit radiation sensitivity in vitro, seem to be very resistant to radiation in vivo, thus suggesting that irradiation may not be a rate-limiting factor for malignant glioma tumor growth.[18]

Colony forming assay is based on colony development of cells which will specialize with stem cells in the tumor cell population. Cancer cells are widely proliferating indefinitely like stem cells. There is increasing evidence that cancers may contain their own stem cells. Many cancers, like normal organs, seem to be maintained by a hierarchical organization that includes slowly dividing stem cells, rapidly dividing transit amplifying cells (precursor cells), and differentiated cells.[19,20] The presence of a small subpopulation of slowly dividing cancer stem cells may elucidate why so many cancers recur after treatment with irradiation or cytotoxic drugs, even when most of the cancer cells seem to be killed by the therapy. Usually, some cancer cells survive the treatment, and these surviving cells may be cancer stem cells, which may be not only resistant to the therapy but also essential for the malignancy of the cancer and malignant cell lines, which have been maintained for years in culture, contain a subpopulation of stem cells.[21]

In this study, we determine the optimal dose of radiation in C6 glioma colony forming assay. Colony forming assay represents three dimensional structural forms of tumors in laboratory conditions and many studies suggest that the human tumor colony-forming assay may be a valuable tool for antitumor treatment screening.[22] Colony forming assay technique was used to determine the appropriate dose of radiation therapy and it was investigated in terms of feasibility, validity, and potential for discovering new antitumor treatment.[23,24] Lu et al showed that clonogenic survival assay offers the potential to study the intrinsic radiosensitivity, repair, long term regeneration, and other radiobiological responses of neural stem cells after in vitro or in vivo irradiation.[25] Colony forming assay with

TLI and BrdU labeling index may be new methods for determining radiotherapy doses in cancer. Indeed, it is possible to investigate these methods in primary cell cultures to determine the appropriate dose in clinical applications. Either human tumor colony-forming assay or other new remedial methods would possibly depend on advances in basic research.

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