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Research the Mechanism of Various Antineoplastic Agents with Use of Flow Cytometry *in Vitro* Glioma Cells

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1. Introduction

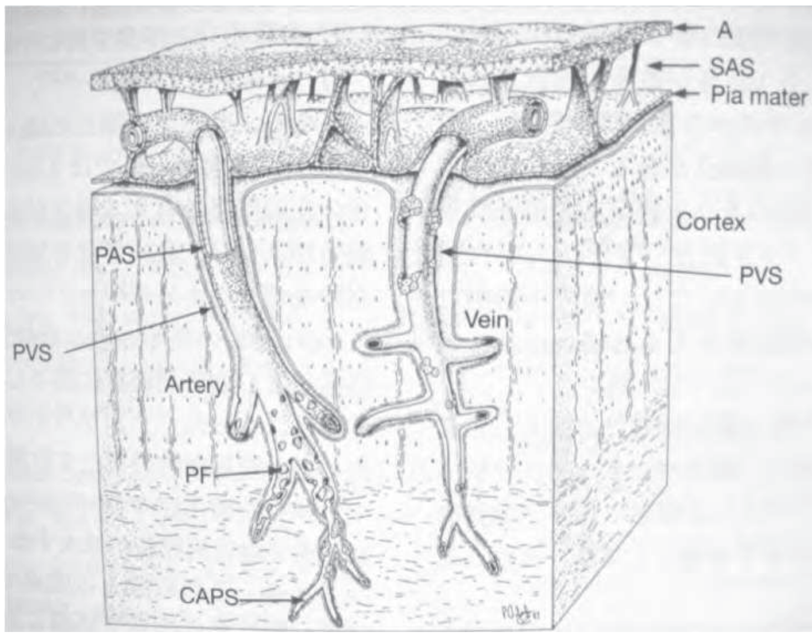
Nowadays, steady progress like in endovascular, microsurgical, neuroendoscopic fields, affect deeply neurosurgergical field, too. But in spite of arising so many innovations, average survival time in glioma is a year and five year survival rate is 8%. These results have not slightly changed for 30 years.(*1)

For us to continue research glioma in future, our purpose discloses what we should be going to do for improve prognosis and needs to analyze data about tumor cells in the various views. In our laboratory, we research brain tumor with flow cytometory. In this chapter, we describe how to analyze the mechanism of various antineoplastic agents for tumor cells centered glioma and research results with flow cytometry.

2. Chemotherapy for central nerves system

There is Blood Brain Barrier, as you know, in central nervous system like a brain and a spinal cord. We can easy to understand the Blood Brain Barrier referred to fig 1(*2). Arteries and veins from subarachnoid space feed the brain after perforating vertically brain pia matter and extending into brain substance. In extending into brain substance, micro perivascular space is formed around vessels in brain substance. It is for us to observe a section of vessels in brain substance (Fig1-2). Vascular endothelium cells adhered to basement membrane form the structure like a tube with tight junction constituted by astrocyte and pericyte adhered outside of basement membrane. This structure formed the group of cells around basement membrane and tight junction between each endothelium around vessels is, so to speak, the Blood Brain Barrier.

The Blood Brain Barrier is hard to absorb aqueous solution. So This is the difference central nervous system from body except it. This property has been the limitation for developing chemotherapeutic drugs. According to same above reasons, chemotherapeutic drugs for glioma also were limited. But, recently, it has produced starting from ACNU, BCNU, CCNU, MCNU, bleomycin , IFN etc, via PCZ, VCR, to TMZ approved. Especially TMZ has been a standard drugs for glioma in the world. Though it gradually has progressed, variation for combination therapy for glioma has increased too. In this time, we analyzed these chemotherapeutic drugs with FCM, LSC as below.



Abbreviation: A: Arachnoid membrane, SAS: Subarachnoid space, PAS: Periarterial space, PVS: Perivenous space, PF: Perforater foramen, CAPS: Capillary artery perispace

Fig. 1-1. Shows blood brain barrier.

Fig. 1-2. Reveal tomography around of micro vessels in the brain.

2.1 Prostaglandin J2α(PGJ2α)

PGJ2α is one of eicosanoid producing in the body. In the beginning of 1980, it cleared up that PGD2 was produced after dehydration need to no enzyme. From the late of 1980 to the beginning of 1990, it was reported the very effective inhibition of tumor cells and viruses. So we expect to have thought of one of new antineoplasitc agent and antiviral drug. But it doesn't attains to apply clinically the glioma therapy, because it is unstable in the body. (*3)

2.2 Interferon (IFN)

IFN belongs to glycoprotein family. IFN has been reported it has direct effect for inhibition tumor growth, indirect effect with immune system and the synergy effect with other antineoplastic agents(*4). Though IFN has subtypes $\alpha \sim \gamma$, as shown our experimental result, IFNβ is the most effective for glioma(*5). But direct tumor suppression effect about IFN is only 18%. In the reports until now, IFN usually has effects combined with other antineoplastic agents. The famous report as for IFN combined with other drugs is about IAR(IFNβ+ACNU+Radiation) therapy. Median survival time about only radiation therapy, radiation+ACNU and IAR therapy were each 15.2 months, 19.7 months and 25.3 months. Initial response rate about radiation +ACNU, IAR therapy were each 35.7%, 60.5%. As this result, IAR therapy showed clearly better result than the other (level 4 evidence) (*28). Response rate in recent report about IAR therapy is 33%(*29).

2.3 ACNU

ACNU is the drug belonging to nitrosourea. This drug easily can pass through BBB. So this was standard drug against glioma until TMZ appeared. Though BCNU, CCNU, MCNU is developed in the West, ACNU is developed and used in Japan. As for metaanalysis, ACNU extended survival time to about one or two year and survival rate increased 6-10% and Median survival time extended to about two months. But this results were unsatisfied with us (*6,7).

2.4 CDDP, CBDCA

Cisplatin isn't general therapeutic drug against glioma.

Rosenberg et al reported it in 1965(*8). CDDP is one of the antineoplastic agent had very wide spectrum for various solid tumors. On the other hand, CDDP has high percentage of side effect like a toxicity of kidney, the digestive system and auditory system. Carboplatin (CBDCA) induced from CDDP was developed in order to reduce the toxicity CBDCA is platinum antineoplastic agent in second generation and was developed in England by Harrap. Though CBDCA has less antineoplastic effect than CDDP, CBDCA reduces clearly side effect like the toxicity for kidney, the digestive system and auditory system(*9).

2.5 As_2O_3 (Arsenic trioxide)

As_2O_3, which is originally used to treat acute promyelocytic leukemia (APL) since the early 1970s at Harbin Medical University in China, has drawn attention to treat solid tumors including gliomas. As_2O_3 enhanced radiation response and increase cure rate of glioma patients.Mechanisms that might explain the anti-tumor cytotoxicity of As_2O_3 include its ability to induce cellular differentiation, tumor apoptosis, the degradation of specific APL transcripts, and inhibition of tumor cell growth by modulating redox balance and/or mitochondrial membrane potential.(*24)

2.6 Temozolomide (TMZ)

Temozolomide belongs to the second generation of alkylating agents, and it can be orally. This drug has become a standard antineoplastic agent against malignant glioma. The effectiveness of this drug has been verified with much evidence. From 1995 to 1997, Yung et al. studied the first endpoint of 6 months without tumor progression in cases of recurrent anaplastic astrocytoma after addition of 200mg/m2 TMZ during the first 5 days. They analyzed about 111 of 162 anaplastic astrocytoma or anaplastic oligodendrocytoma cases diagnosed in their pathology center. As a result of these analyses, over all response rate (RR) is 66%; for anaplastic astrocytoma, RR is 62%. The complete response rate is 6% and the partial response rate is 28%. At present, relatively good outcomes are ensured and have been accumulated in phase studies such as those by the RTOG Group. One of the representative phase Ⅲ study is the research that ,so to speak, Stupp regimentation was added against cohort of 573 cases caught initially GBM by EORTC (European Organization for Research and Treatment of Cancer) and NCIC(national cancer institute of Canada). In summary, induction therapy is GBM after tumor resection is irradiated 60Gy and given TMZ (75mg/m2/day) for 6 week. Maintenance therapy is they gave 6 courses, when one course is TMZ (150～200mg/m2/day) for 5 days on and 23 days off. In the result from this comparison only irradiation with this regimentation, MST (median survival time) is 14.6 months in 247 cases administrated TMZ and irradiated, 12.1 months in 286 cases only irradiated. 2 year's survival rate is 26.5% in TMZ group, 10.4% in irradiated group. Hazard ratio for death is 38% statistically dominant decrease. In spite of excellent result like these, incidence of blood toxicity was only 7%.TMZ prized a level Ⅰ evidence.

As above, we used flow cytometer and laser cytometer for tools of analysis the drugs for pharmacological mechanism already known on the basis of cell kinetics.

3. History of flow cytometry (Fig2) and laser cytometry

Flow cytometer was developed for cancer research in Los Alamos institute in 1959, simultaneously, for immune system research in Stanford University. Then, various flow cytometer are developed as fig2. Recently, a serious of FACS made in BD corporation and Epix made in Coulter company and so on are familiarized. These developments caused we can measure amount of various kinds of ingredients like intracellular DNA and of protein, can easy to analyze cell cycle too. In addition, it could see, measure amount of antigens on cell surface, major progress with immunology field.

On the other hand, it had developed image cytometry of analyses about cell figures and so on. In 1976, Kawamoto in our institution started the measure intracellular DNA of brain tumors, in his being in Monte Fiore Hospital in New York. In 1976, he used with the prototype of Fluorograph (Fig3-1), which could measure amount of DNA and its histogram, not analyze cell cycles. In 2011, we use, in our institution, FACS Calivur (Fig3-2), which can analyze simultaneously and automatically histogram, cell cycle, DNA index (DI) calculate. In 1991, Kamentsky et al invented Laser Scannning Cytometer(LSC) which was the machine having dual character of FCM and image cytometory. LSC can analyze automatically amount of DNA and grasp cell figures, after put above the slide prepared for a microscope.

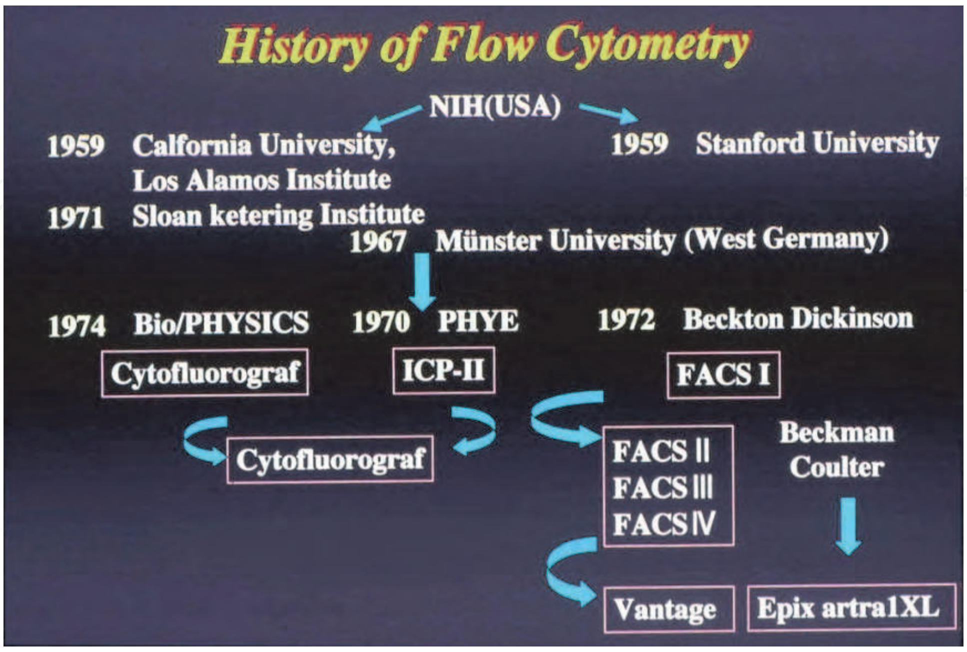


Fig. 2. History of Flow Cytometry

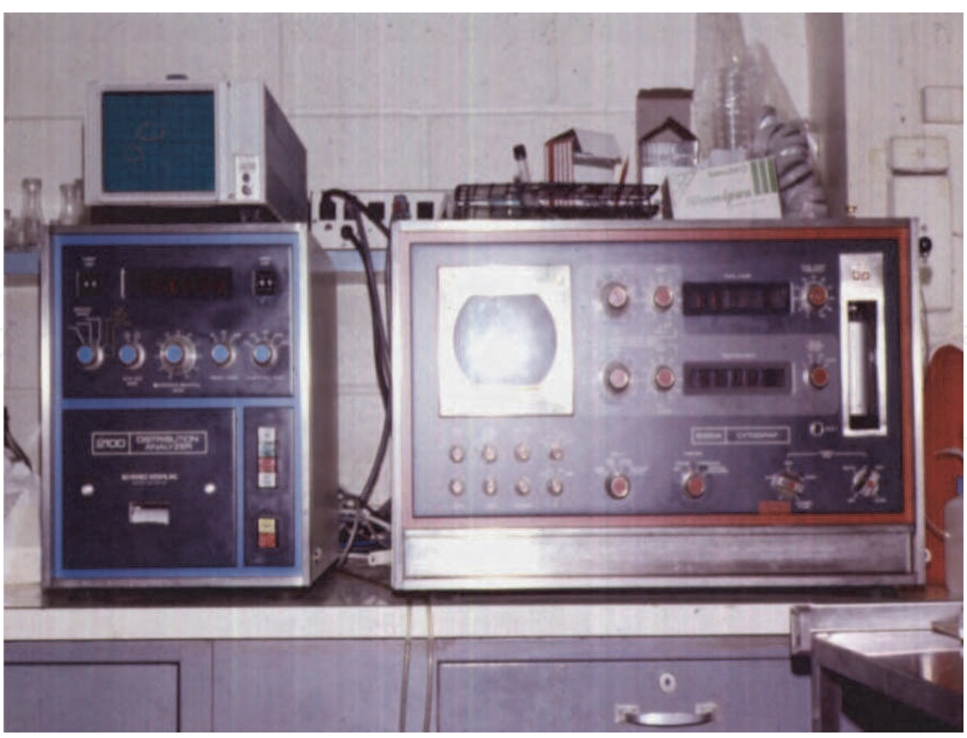


Fig. 3-1. Prototype of Fluorograph could measure amount of DNA and its histogram, not analyze cell cycles (in1976, Monte Fiore Hospital, New York).

Fig. 3-2. FACS Calivur in our institution can analyze simultaneously and automatically histogram, cell cycle, DNA index (DI) calculated .

4. The principle of flowcytometry (Fig4)

Isolated cell stained with fluorescent pigment dropped from the nozzle. After laser hit each cells, it can identify fluorescence dividing into two directions. These fluorescences are frontal scatter fluorescence meaning cell size (figure) and lateral scatter fluorescence meaning biological property (=relative amount of DNA). Sensors on each direction translate these intensities into electric signals depending on amount of DNA, and conduct electric circuit (*10). We can analysis passing these signals from 1000 to 5000 cells per second with computer. More advanced machine can sort cells through making dropped cells plus or minus charge, so to speak, cell sorting (*11,12). Because these machines are delicate, we have to adjust repeatedly for reliable datum. Mainly, these adjustments include flow system, the axis of laser. It is important to line up stable laser and water pressure for continue to flow isolated cells with regular speeds and orderly from the nozzle. As it repeats the adjustments on two or three times with, for example, micro beads, calculates coefficient of variation: CV of histogram. In this time, CV with micro beads hopes to be less than 2%. About how to adjust in detail, you had better refer to texts and papers (*13,14)

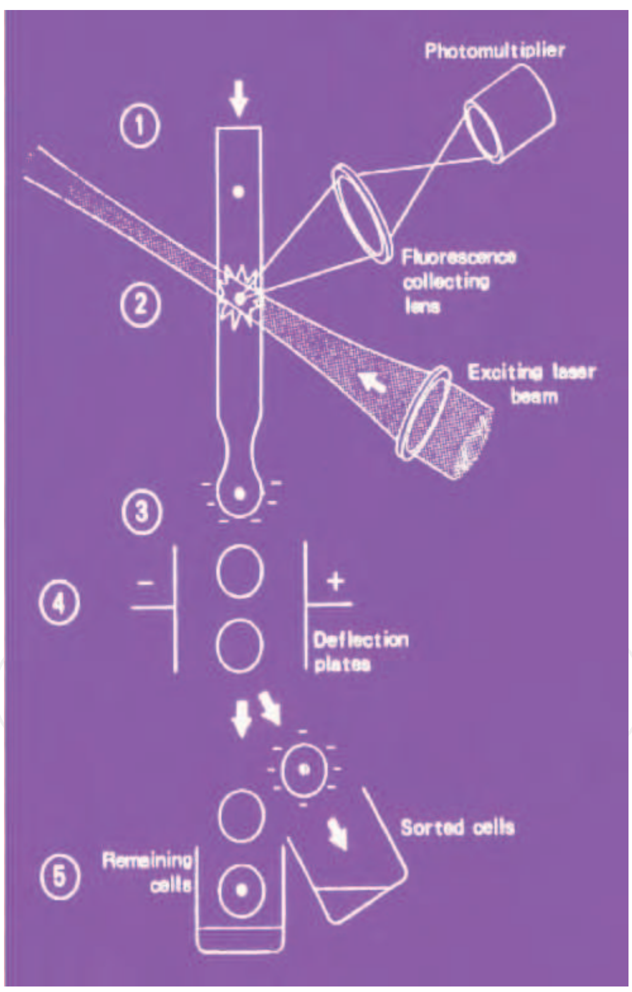


Fig. 4. Figure reveals principle of flow cytometory. Isolated cell droped from the nozzle. Laser hit each cells. We can analysis passing this laser from 1000 to 5000 cells. More advanced machine can sort cells through making dropped cells plus or minus charge. This technique can choose cells we can point out free.

5. About scattergram (dotgram)

The data analyzed with flowcytometer good adjusted reveals a scattergram (dotgram) like fig 5. Scattergram (dotgram) is consist of x axis set frontal scatter concentration of fluorescence and y axis set lateral scatter concentration of fluorescence, is the aggregation result from plotting on the basis of each value. Because this aggregation includes debris of cells and unnecessary cells, we need to narrow moreover aiming cells. What we do to narrow aiming cells is called gating procedure. Gate is the procedure sort a aiming group of cells on the basis of wavelength of beam, from whole group of cells. Fig 5 reveals dotgram consisted of X axis set 7-AAD used in measuring amount of DNA and Y axis set BrdU used in measuring amount of cells in S phase. Revealing concretely with Fig 5, the procedure of gating is surrounding square each group of cells. If it sets up gating on same condition, you can measure the percentage of any cell groups in whole of objective group (In fig.5, you can measure how percentage of each cell in each cell cycle). In addition, if you stain cells with monoclonal antigen with FITC (BrdU with FITC in fig5), can response differentially antigen on cell surface, you can measure quickly and objectively not only whether antigen stained with fluorescence or not, but also measure that densities.

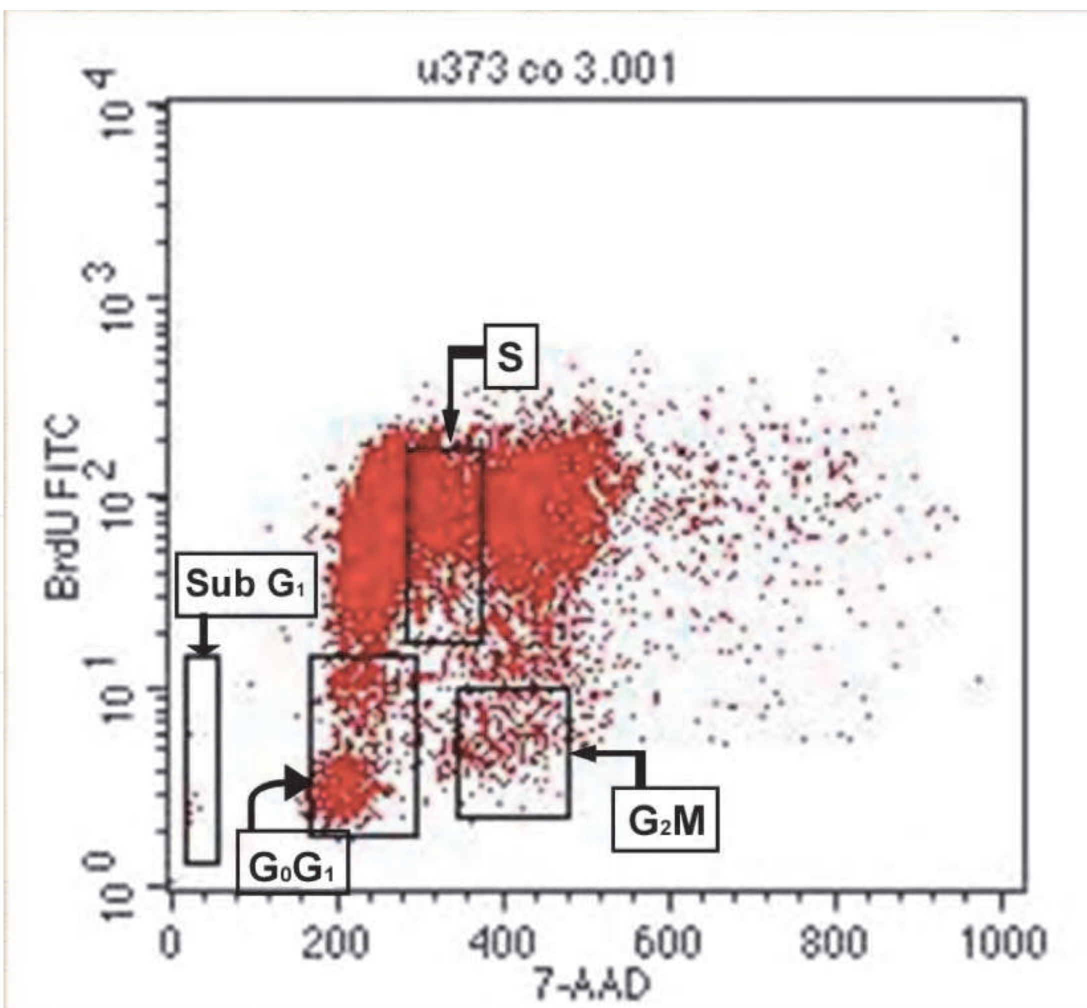
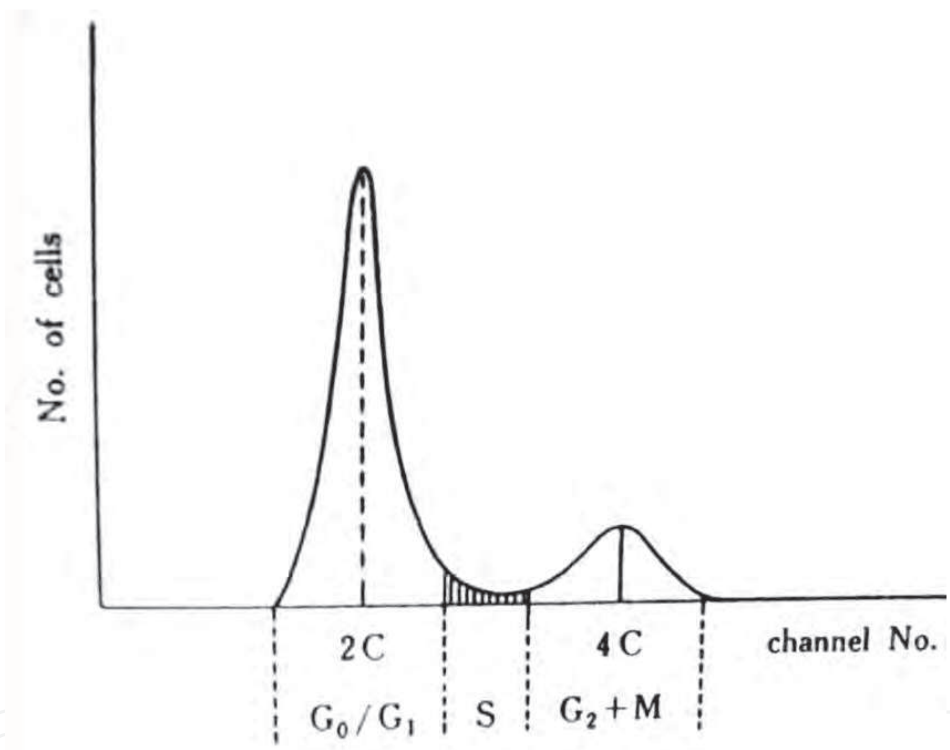


Fig. 5. This is the dotgram consisted of X axis set 7-AAD and Y axis set BrdU with FITC.

6. About histogram

Various kinds of fluorescence stain are used in measuring amount of nuclear DNA. It is hoped that these amount of fluorescence pigment in linear proportion to amount of DNA. In our institution, we use PI (Propidum iodide), 7-AAD (Actinomycin D). As s result of measure amount of DNA depending on amount of fluorescence pigment, it reveals DNA histogram. Generally, there are G_0G_1 phase cells having DNA amount of 2C, G_2M phase cells having DNA amount of 4C and S (=synthesis) phase cells between G_0G_1 and G_2M phase. Because in S phase the reason of overlap a group of cells in early S phase with a group of cells in G_0G_1 phase and the reason of overlap a group of cells in late S phase with a group of G_2M phase, we have difficulty in identifying a group cells in S phase. Various mathematical models for calculate each division of S, G_0G_1, S, G_2M on cell cycles, were reported. For example, Baisch.H et al(*15), Bariogie B et al(*19), Fried et al(*17,18) were reported. In this time, we adopted three-division method (Kawamoto et al) (*16) (Fig6).



First peak, G_0G_1 phase; second peak G_2M phase; between two peaks, S phase

Fig. 6. The three-division histogram method (Kawamoto et al.).

7. Clinical material and method

We used the target cells as follow: human glioma cell lines like U373, U251, U87MG, KMU100, rat glioma cell lines like 9-LMG, C6 and KB cell line (nasopharyngeal carcinoma. Depending on each experiment, we added each cell line to medium concentration dose near ED50 (effective dose 50%) and high concentration dose more than ED 50. ED50 was decided on the basis of result from basic experiment like phase Ⅰ study. Cell killing effect depending on concentration of dose was assessed with cell count depending on process of times and days. Simultaneously, we analyzed the histograms with FCM (or LSC) depending on process of days. When we make the specimen for FCM, we can easy to understand in sight through sometimes using double staining (BrdU, PI, 7AAD etc.) method depending on process of days. Drugs our analyses histogram in this chapter are ACNU, IFN, PGJ2α, DCCP, A2O3, TMZ.

7.1 Culture method

The established cell lines were subjected to monolayer culture in minimum essential medium (MEM) and dMEM(Dulbecco's modified Eagle's medium)(Gibco:high glucouse with L-glutamine with pyridoxine hydrochloride without sodium pyruvate or sodium bicarbonate) supplemented with 10% fetal bovine serum(FBS) in a 5% CO_2 incubator.

7.2 How to make specimen for FCM

A single suspended cell according to method our draw above was fixed by 70% ethanol. After it was made reaction of 0.5% RNAse treatment under 37℃ for 30 minutes, 7AAD or PI staining treated cells went into FCM owing to measure amount of DNA. Flow cytometers we used were FACStar and FACS Calivur supported by Becton Dickinson (BD) Corporation. For example, FACStar's laser has wavelength of 488nm, wave strength of 200-500mW and long pass filter of 520nm.

7.3 Double staining method

For example, in the case that you want to ensure specifically S phase cell, you make reaction monoclonal antibody to BrdU with IgG labeled FITC. Everyone has understood DNA uptake BrdU (Bromodeoxyuridine) as thymidine (*20). After monoclonal antibody ensured BrdU which was developed by Gratzner et al (*21) in 1982, we can analyze cell growth with BrdU. Though BrdU is taken into intranuclear DNA in synthesis (S) phase, we can identify cells taken anti BrdU antibody by stain after short time treatment. If Z axis are set the density of dots in dotgram in addition to X axis set PI or 7AAD and Y axis set BrdU, we can get 3D expression of histogram. 3D histogram makes us easy to sight cell distribution in S phase (fig7).

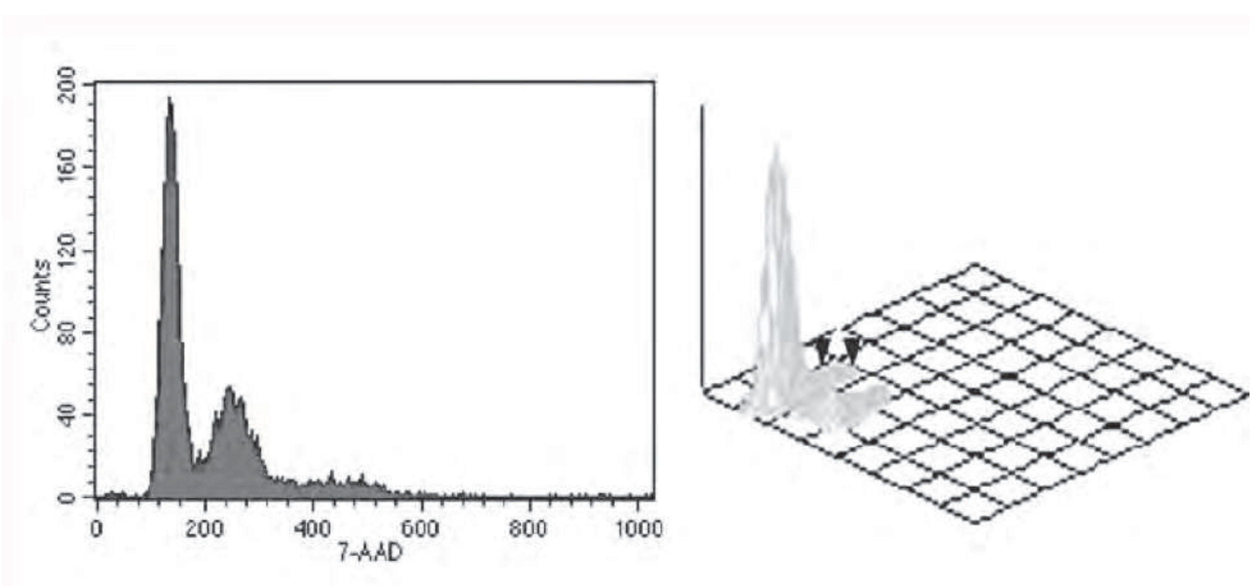


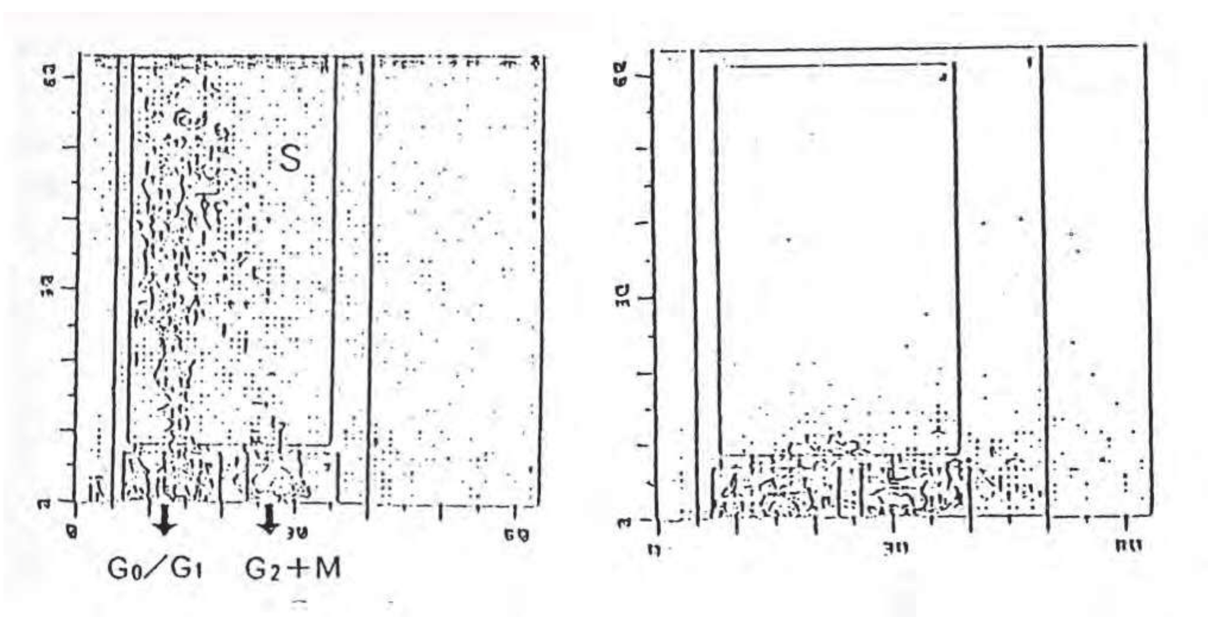
Fig. 7. Example of exchange 2D (left graph) for 3D expression (right graph) It is hard to identify S-phase cell accumulation with 3D expression (arrowheads)

On the other hand, when you want to check the cell viability, you had better stain with Fluorescein diacetate (FDA). Like these examples, when it identifies specifically the relationship between cell cycles and cell distributions, this technique is inducted.
Studies and results about each antineoplastic agents.
Summary of our results are below.

1. PGJ2 blocks cell cycle at G_0G_1.
2. IFN 、ACNU 、CDDP、As_2O_3 、TMZ block cell cycle at S phase.
3. ACNU、CDDP、As_2O_3、TMZ blocks at G_2M phase.

7.3.1 Prostaglandin J2α(PGJ2α)

We tried to analyze the mechanism of PGJ2α with FCM after addition for PGJ2α to tumor cells(Fig8). The cells distribution in S phase are disappeared after addition for PGJ2α to tumor cells. In other words, cells accumulate in G_0G_1phase and G_2M phase. So PG is effective to G_0G_1 phase.



Left graph is control. Right graph is in case of add PG. Cells in S phase are disappear and cells accumulate in G_0G_1, G_2M phase. Results from the above, we thought, PGJ2α effect for GoG1 phase.

Fig. 8. Reveals dotgram meaning the mechanism of PGJ2α's antineoplastic effect.

7.3.2 Interferon (IFN)

In the case of IFN, after we added each α, β, γ IFN(low～high dose like 10^2～10^5IU/ml) to U373MG(10^5/dish), counted cells on 1st ,3rd,5th day. Results of that, we studied the suppression effect of propagate. When studied the graph about cell count after addition of IFN α, β, γ as figure 9, we can observe the suppression of cell count depending on the IFN concentration .

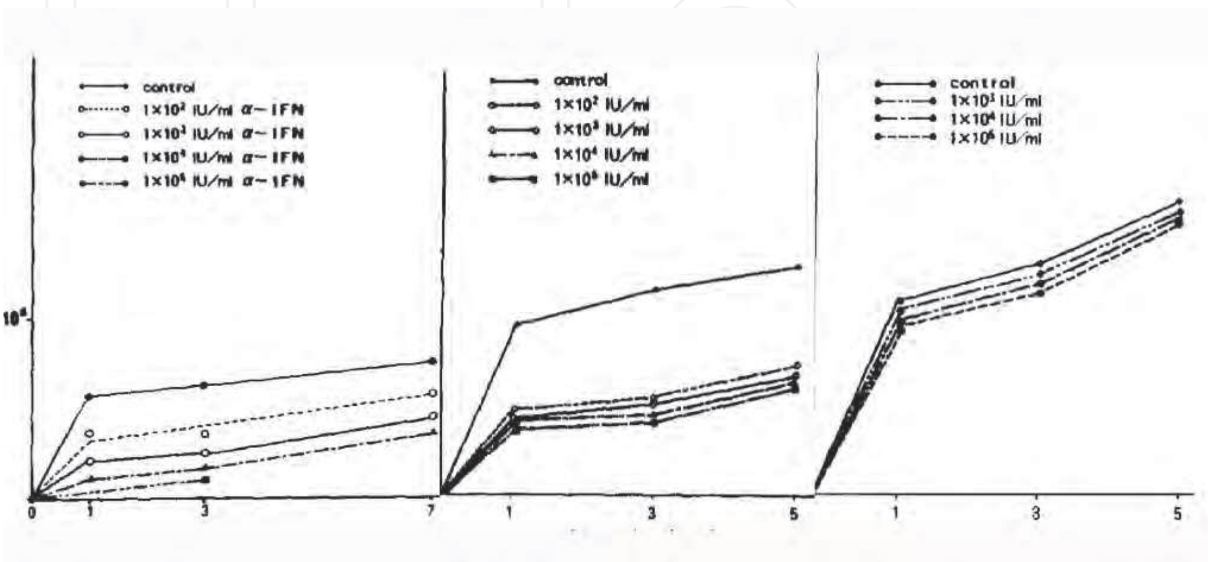


Fig. 9. Graphs from left to right reveal graph of α,β,γ. Concentrations are heighen increasing from upper to lower. The drug having the biggest subtraction from control was IFN βwhich had the highest suppression of propagation. IFN α had second suppression of propagation. In this experiment, IFN γ has not almost suppression of propagation.

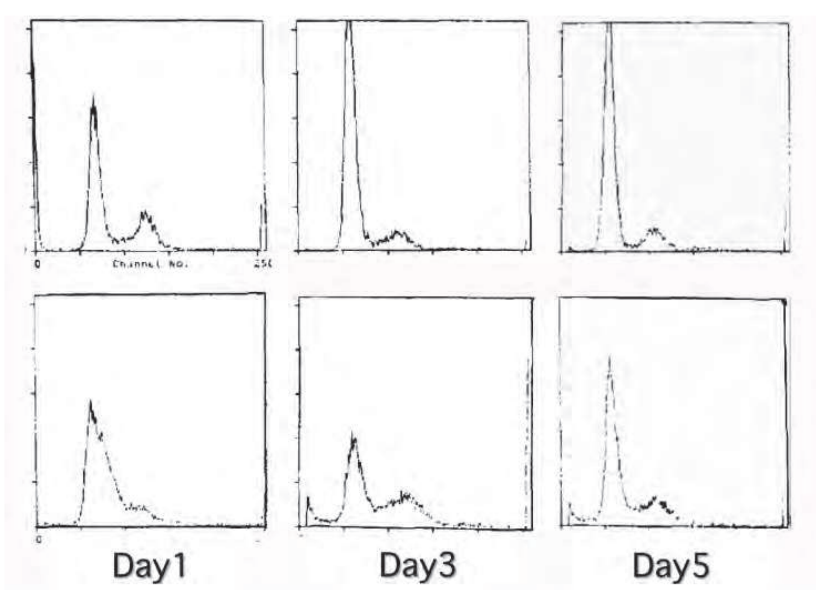


Fig. 10. Upper row is control. Lower row is histograms of IFNβ. IFNβ has early S phase block earlier than control group. Then, S phase block was clarified on 3rd day. On 5th day, it was observed recruitment.

The drug having the biggest subtraction from control was IFN βwhich had the highest suppression of propagation. IFN α had second suppression of propagation. In this experiment, IFN γ has not almost suppression of propagation. As a result of this, we studied cell kinetics with IFNβ. After 104IU/ml IFNβ was added to U373MG(105/dish), we counted cells on 1st ,3rd,5th day and analyzed histogram with FCM. These results are like Fig10. IFNβ has early S phase block earlier than control group. Then, S phase block was clarified on 3rd day. On 5th day, it was observed recruitment.

7.3.3 ACNU

It reveals a DNA histogram resulted from the addition 5μg/ml 、10μg/ml ACNU to U251MG cell lines(fig 11). Standard histogram is revealed in the group of control without the addition ACNU. The histogram a day after addition 10μg/ml ACNU reveals DNA accumulation in S phase meaning S phase block. Moreover, histogram two days after addition 5μg/ml ACNU reveals DNA accumulation in G_2M phase and these cells were dead. So ACNU has effect both S and G_2M phase.

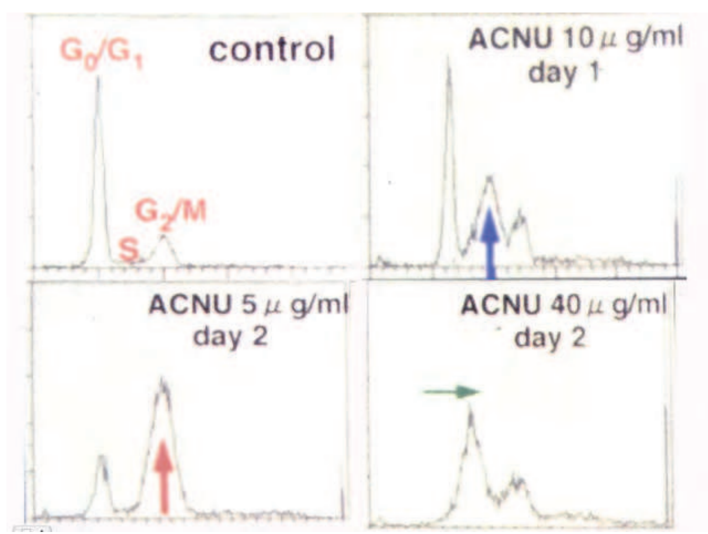


Fig. 11. Analysis of cell cycle (U-251). Left upper:Standard histogram is revealed in the group of control without the addition ACNU. Right upper:The histogram a day after addition 10μg/ml ACNU reveals DNA accumulation in S phase meaning S phase block. Left lower: Histogram two days after addition 5μg/ml ACNU reveals DNA accumulation in G_2M phase and these cells were dead. Right lower: Histogram two days after addition 40 μg/ml ACNU reveals no accumulation meaning recruitment.

7.3.4 CDDP, CBDCA

After it contacted KB cells with 2μg/ml thought ED50 for KB cells CBDCA for 24 hours or with 0.5μg/ml CDDP for 24 hours, KB cells were rinsed with PBS two times. It continued cell culture and analyzed with FCM, in case of CBDCA on 1st, 2nd, 3rd, in case of CDDP on 1st, 3rd, 5th, 7th day. In addition, it was performed double staining with BrdU and 3D analysis. In case of CBDCA, the peak in S phase of cell accumulation on 1st day transferred into the peak in G_2M phase of cell accumulation on 2nd and 3rd day (fig12). In case of CDDP, the peak in S phase on 1st, 3rd day transferred tended to migrate gradually into G_2M phase according to the progression like 5th, 7th day (fig13).

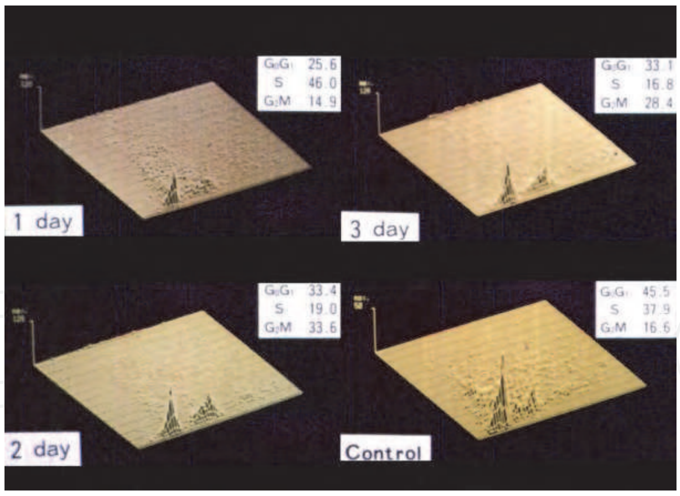


Fig. 12. 3D expression of Carboplatin reveals the peak in S phase of cell accumulation on 1st day transferred into the peak in G_2M phase of cell accumulation on 2nd -3rd day.

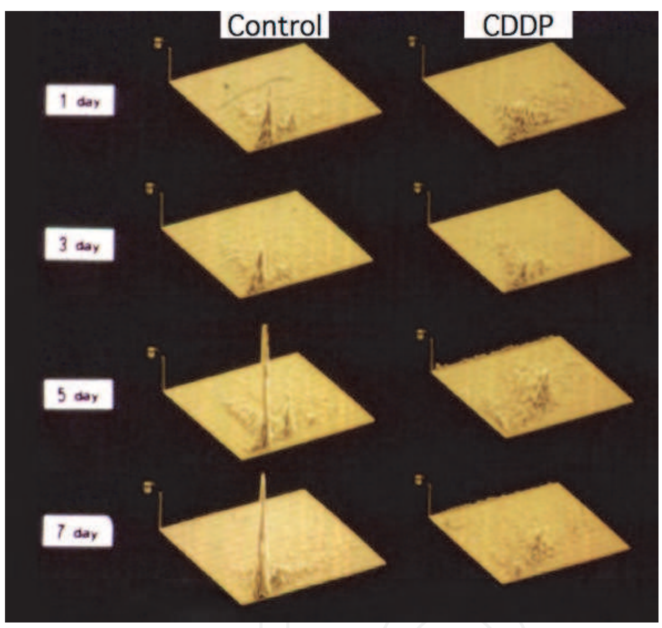


Fig. 13. 3D expression of cisplatin reveals the peak in S phase on 1st, 3rd day transferred tended to migrate gradually into G_2M phase according to the progression like 5th, 7th day .

Result from above, CBDCA has effect in S phase and G_2M phase. In case of CDDP, it has the same tendency. So CDDP has also effect in S phase and G_2M phase (*22)

7.3.5 As_2O_3

It analyzed the change depending on passing time after the addition As_2O_3 to U87MG and T98G cell lines with LSC (fig14).
In case of U87MG, it observed a DNA accumulation tendency in S phase until 24 hours from the addition, this peak immigrated into G_2M phase from 24 hours to 72 hours and it observed slight accumulation in sub G1 phase from 24 hours to 48 hours. Though the same tendency was in case of T98G, this tendency of cell accumulation was slight different from U87MG like fig 14. Result from the above, As_2O_3 has effective in S and G_2M phase (*23).

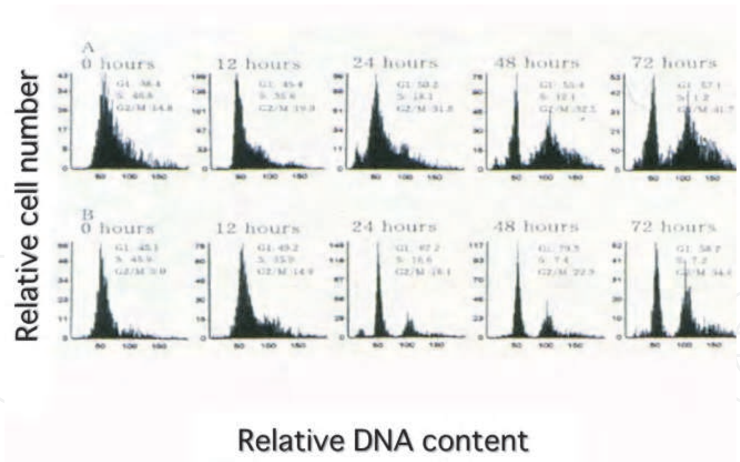


Fig. 14. Cellular DNA content frequency histograms demonstrating As_2O_3-induced changes in cell cycle distribution and apoptosis of U87MG(A) and T98G(B) cells.

7.3.6 Temozolomide(TMZ)

Each U373MG (human glioma cell line), U87MG (human) and 9L (rat) cell line was divided into three groups: a control group, a low-dose temozolomide group [addition,100～200 μg/ml temozolomide; near ED 50], and an high-dose temozolomide group [addition, 300～500 μg/ml]. On day 1, temozolomide was added to each cell line. Then, we counted the number of cells on days 2,3,4 and 5. In the U87MG line, we counted the number of cells on days 8 and 9. Simultaneously, we performed flow cytometric analysis with the double staining (7-AAD and BrdU)(fig 15).

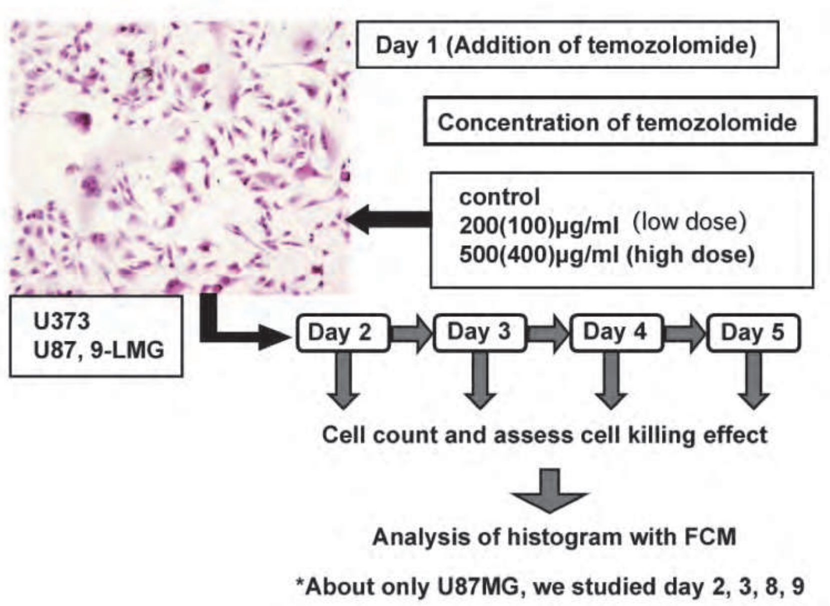


Fig. 15. This is experimental method about TMZ. Each cell line was divided into three groups, control group, low-dose TMZ group, high-dose TMZ group. On Day 1, TMZ was added to each cell line. Then, we counted the number of cells on Day 2, 3, 4, and 5. In U87MG, we counted the number of cells on Day 8 and 9. Simultaneously, we performed flow cytometric analysis with single and double staining methods.

7.4 Result 1

Suppression of cell proliferation with TMZ

Growth curves of all cell lines showed suppression of cell growth depended on TMZ concentration. A 50% cell-killing effect was obtained with 200-500 μ g/ml TMZ in U373 and 100-400 μ g/ml TMZ in U87MG and 9LMG. This concentration is considered the ED50 (fig16) In the U373MG cell line, a significant decrease(χ square test) in the growth curve was observed with 200 μ g/ml and 500 μ g/ml TMZ on day5. In the 9-LMG cell line, a significant decrease was observed in 100 μ g/ml and 400 μ g/ml TMZ after day 3.Tumor cells (in all cell lines) to which TMZ was added showed morphologically shrinkage of cell processes, ligetening of the nucleus and cell atrophy (fig 17).

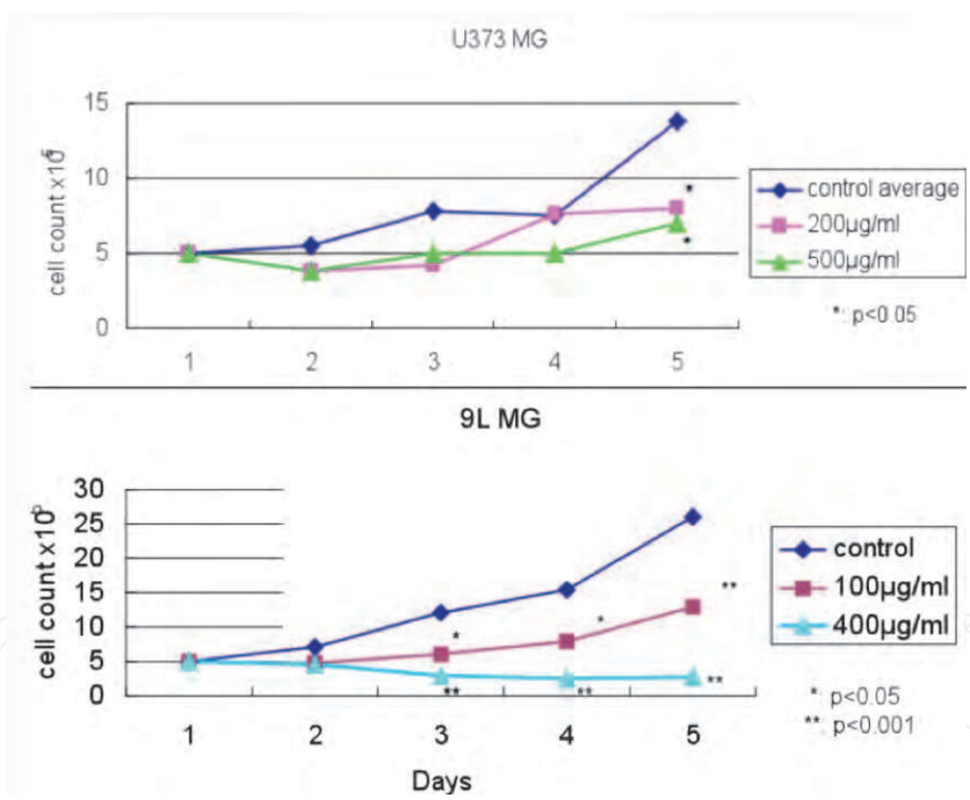
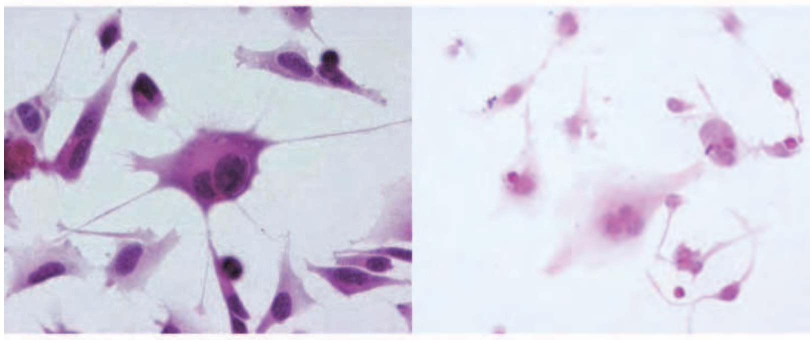


Fig. 16. Results of cell counts for U373MG and 9LMG. Both cell lines show that the cell killing effect depends on dose concentration.



Left: normal glioma cells (before TMZ addition)
Right:Glioma cells after addtion of TMZ show shrinkage of cell processes pale nuclei, and call atrophy

Fig. 17. Morphological change of U373 cells with hemaatoxylin and eosin (H&E) stain before and after addition of TMZ

7.5 Result 2

Analyses of effect of TMZ on cell cycle with FCM: The U373MG cell line had a tendency to accumulate in the G_0G_1 and S phases on day2 though 4. On day 4 in the group with 200 μg/ml TMZ added, or days 1 through 4 in the group with 500 μg/ml TMZ added, we observed significant accumulation (fig 18-19). On days 4 though 5, there was a tendency for cells to accumulate in the G_2M phase. Especially on day 5, for 200 μg/ml TMZ and on days 4 through 5, for 500 μg/ml TMZ we observed significant accumulation (see fig19-1,2 and fig 18-3). The 9-L cell line also accumulated in the S phase from days 2 through 3 and then accumulated in the G_2M phase (see fig 19-3). The U87 MG cell line also accumulated in S phase on days 2 through 3, and then accumulation in G_2M phase, and finally in sub-G1 phase on days 8 through 9(see figs 18-5,6)

Result 3: The dominant morphological changes observed in U87MG were confined to the nuclei (fig 20), with positive terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TINEL) staining. These changes suggested apoptosis (*25).

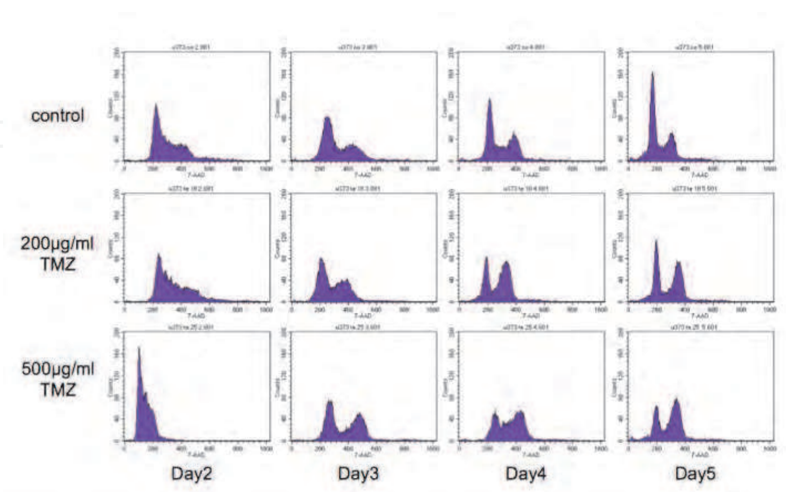


Fig. 18-1. Histogram for U373MG shows changing amount of DNA after addition of TMZ. By this histogram, we can see the G_2M-phase block after S-phase block. In addition, it shows clearly that the G_2M-phase block depends on TMZ concentration.

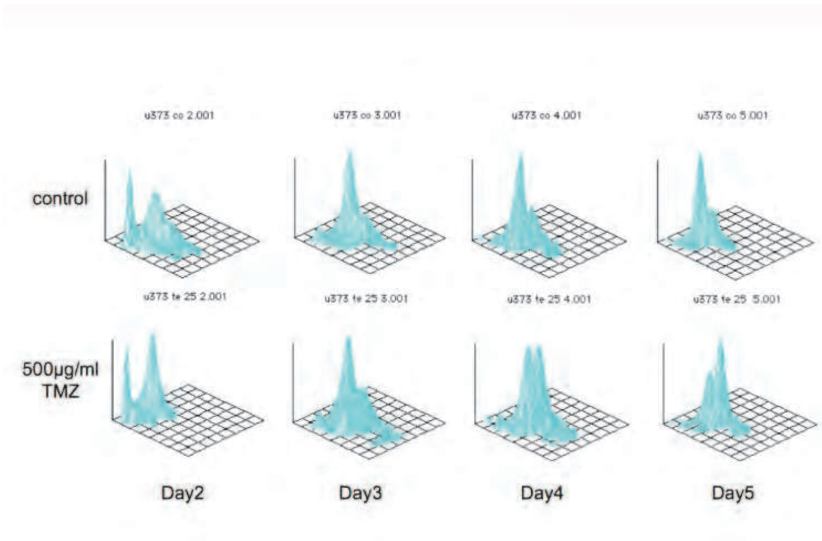


Fig. 18-2. For U373MG: 3D expression of changing amount of DNA by using TMZ. The G_2M-phase block after S-phase block is easily seen

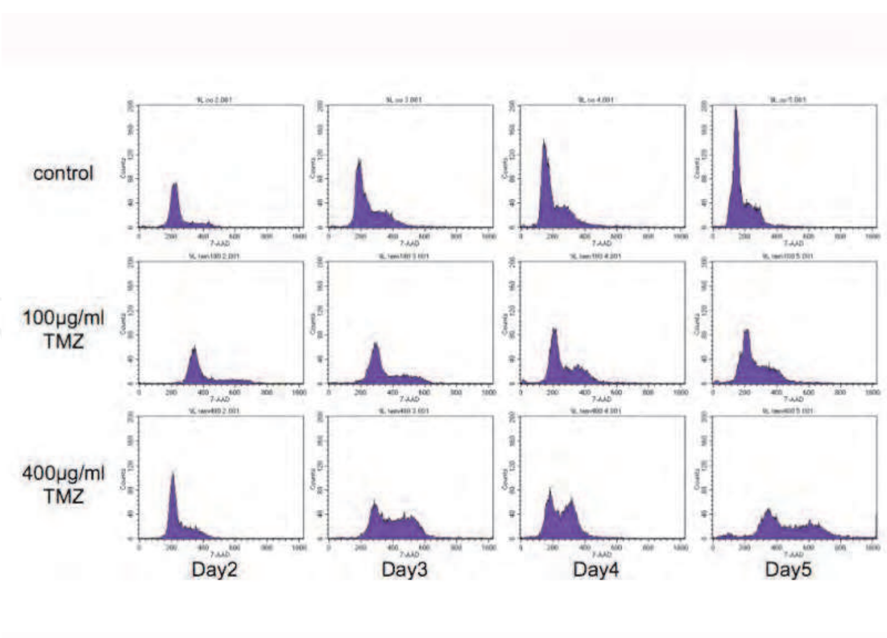


Fig. 18-3. Histogram for 9L MG shows changing amount of DNA after addition of TMZ. By this histogram, we can see the G2- phase block after S-phase block. In addition, it shows clearly that the G_2M-phase block after S-phase block depends on TMZ concentration.

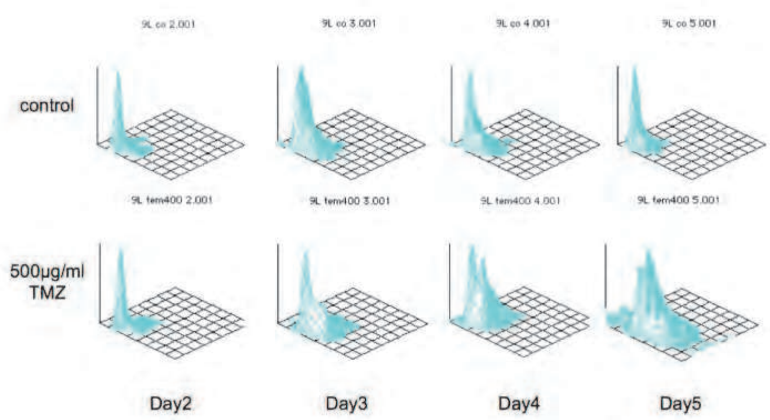


Fig. 18-4. 9LMG: 3D expression of changing amount of DNA by using TMZ. The G_2M – phase block after S-phase block is easily seen.

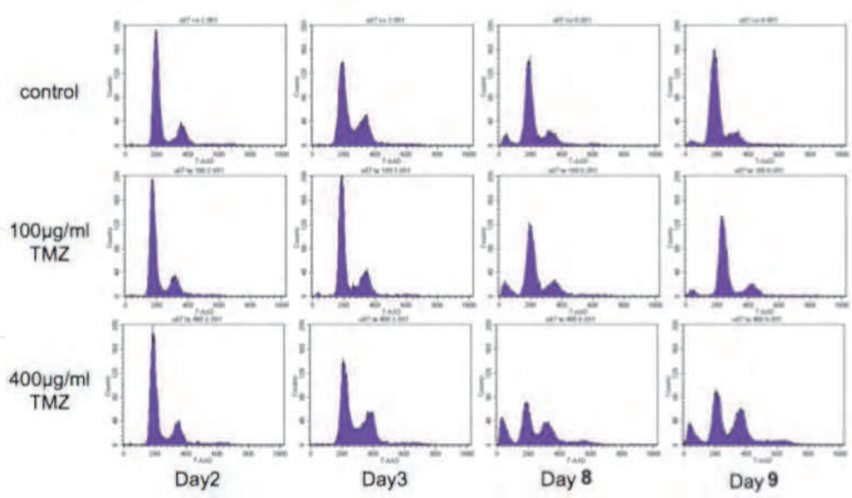


Fig. 18-5. Histogram for U87MG shows changing amount of DNA after addition of TMZ. The sub-G1 phase peak after S-phase block is easily seen.

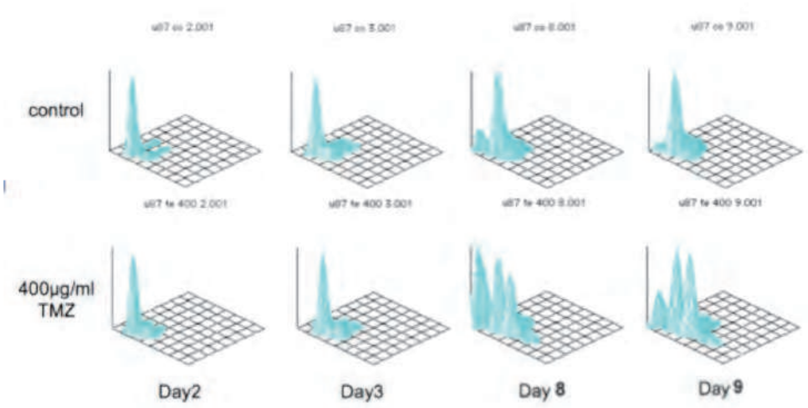


Fig. 18-6. U87MG: 3D expression of changing amount of DNA by using TMZ. The sub-G1 phase peak after S phase block is easily seen.

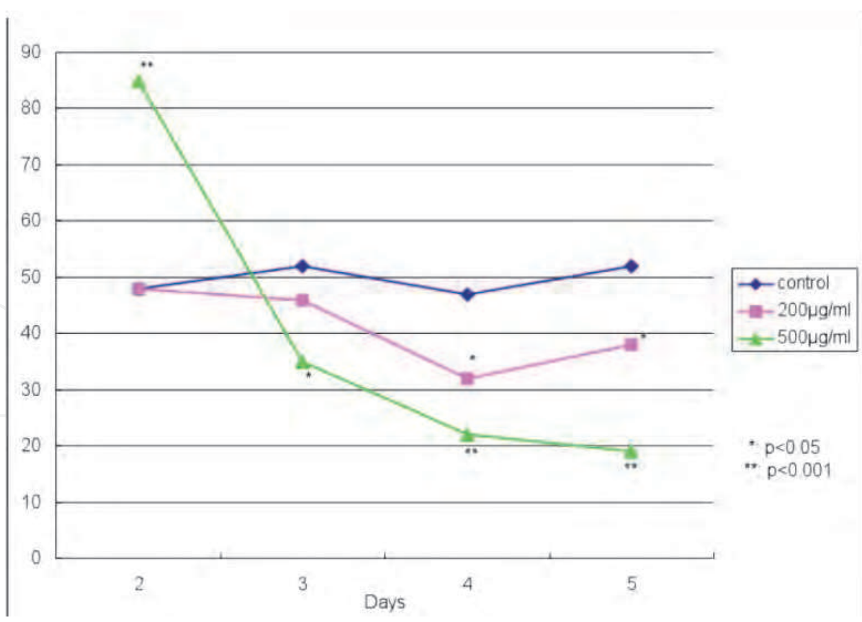
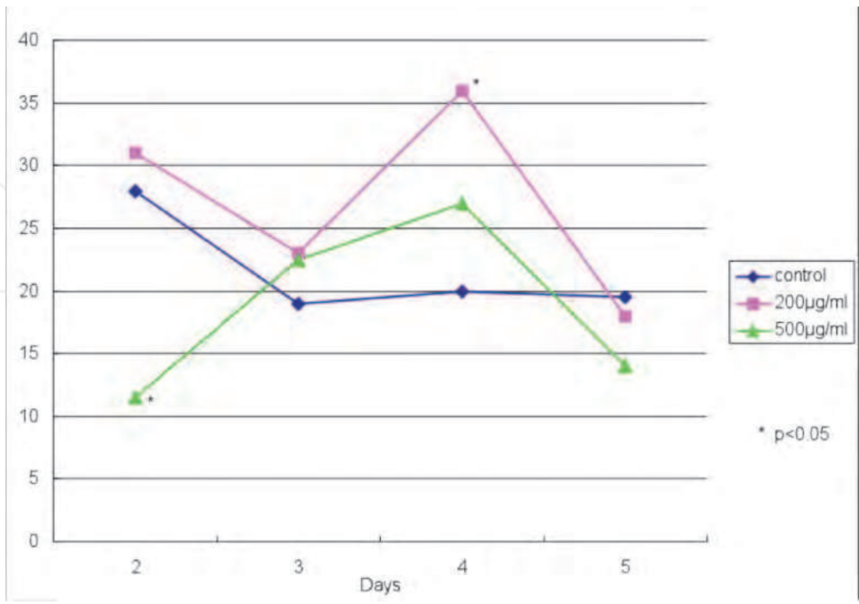


Fig. 19-1. Amount of DNA in G_0G_1 phase with U373MG. On day 2 of addition of 500μg/ml TMZ, we observed a statistically significant increase of DNA. On days 4 and 5 of addition of 200 and 500μg/ml TMZ, we observed a statistically significant decrease of DNA. We called this pattern left peak.



We called this pattern center peak.

Fig. 19-2. Amount of DNA in S phase with U373MG. On day 2 of addition of 500 µg/ml TMZ., We observed a statistically significant decrease of DNA. On day 4 of addition of 200mg/ml TMZ., we observed a statistically significant increase of DNA.

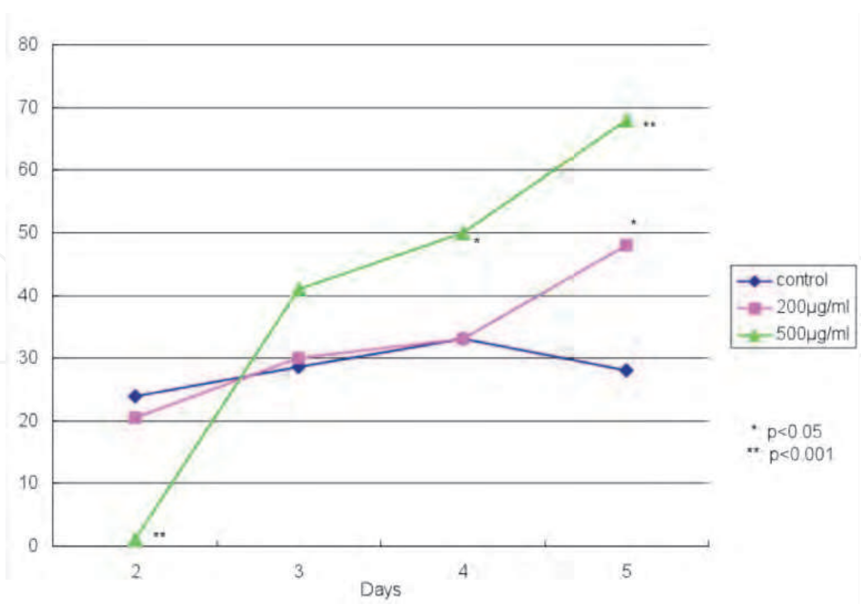


Fig. 19-3. Amount of DNA in G_2M phase with U373MG. On day 2 of addition of 500µg/ml TMZ, we observed a statistically significant decrease of DNA. On days 4 and 5 of addition of 500µg/ml TMZ and 200µg/ml TMZ, we observed statistically significant increase of DNA. We called this pattern right peak.

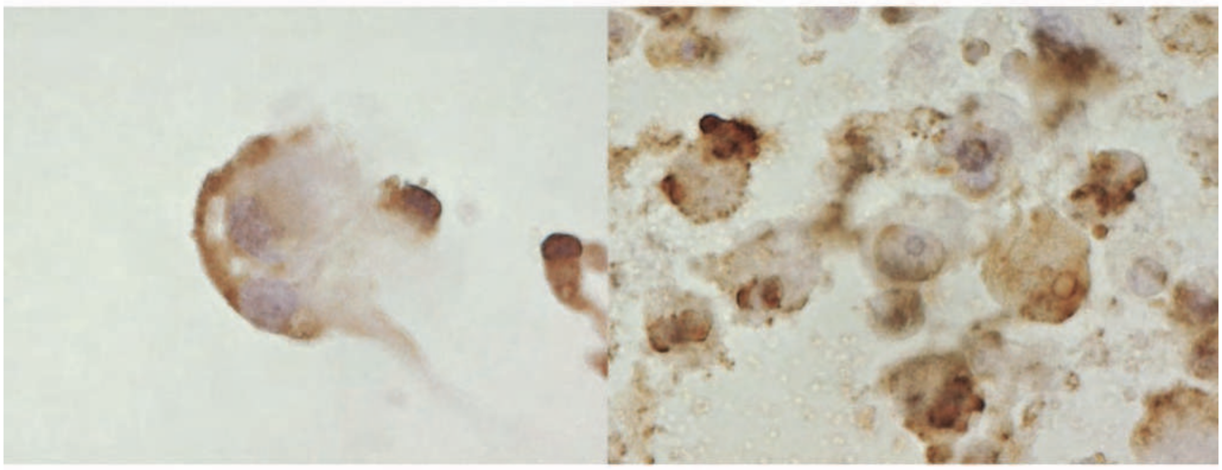


Fig. 20. Apoptotic cells shown by TUNEL stain (left ×600; right×400)

8. Discussion

Each antineoplasitic agent has each pharmacological mechanism. The all reasons of tumor cell killing weren't explained from view in cell kinetics. However, because we can imagine the strategy of treatment on the basis of cell kinetics about each antineoplastic agent, it is truth that information about cell kinetics has important profile.

For example, as the radiation that was used with various types of antineoplastic agents, it has reported the result of analyses from the view of cell kinetics. Though Yokoyama et al reported the slight accumulation in G_2M phase at 24 hours after low dose radiation like 5Gy, 7Gy, it is no subtraction at 48 hours after radiation. We considered this reversible change. But, in case of radiation more than 10Gy, this accumulation in G_2M phase decreased additionally. We considered this tendency irreversible change (*26). Especially, in case of a study about the radiation more than 15 Gy, it reported almost complete G_2M block our considered. In addition, Kubota et al, with HeLa cells, reported the observation of considerable cell accumulation in S phase at 5 hours after 10Gy radiation, then, that accumulation in S phase migrated quickly G_2M phase meaning considerable G_2M block (*27).

As a result of above, low dose radiation made reversible effect and weak cell cycle block, and high radiation made irreversible effect, you know, cell cycle block in S phase migrate in G_2M phase. These results from radiation are similar to the result from drugs like some antineoplastic agents having constant clinical effect. In other word, drugs having weak clinical effect have reversible tendency and showed partially cell cycle block.

What is important in analyze experimentally these cell kinetics, set the cell line and the concentrations of drugs. If it is same antineoplastic agent, these drugs tend to have different results depends on the difference from kind of cell line, drug concentration, addition methods. From the view of clinical application, we might have better choice proper how to add and proper drugs.

For example, as my mentioned before, though S phase block was observed in the result from the addition only IFN to glioma, such block effect tended to reversible. However, the clinical studied effect for glioma cell with using IFN with TMZ is ensured (COG:Japan Clinical Oncolgy Group 0911). In the view of cell kinetics, those mechanisms were considered S and G_2M blocks in radiation, in addition to the synergy effect between temporary S phase block in IFN and S and G_2M blocks in TMZ. From now on everyone expects these effects that IFN made the sensitivity of TMZ up via p 53 via the suppression of MGMT gene, in the view of pharmacology.

It might be hard to ensure, even if experimentally, clinically and also in view of cost, these synergy effects between radiation and antineoplastic agents. However, when we consider the combination of therapeutic alternations and additional methods, we concluded that the study of cell kinetics with FCM and LSC, one of data considered like these, is very effective.

9. Abbreviation

ACNU (nimusutine hydrochloride) ,
BCNU (1,3-Bis (2-Chloroethyl)-1-Nitrosourea),
CCNU(1-(2-chloroethyl)-3-cyclohexyl-l-nitrosourea),
MCNU,(1-(2-chloroethyl)-3-(trans-4-methylcycloethyl)-1-nitrosourea),
IFN (Interferon),
PCZ(Procarbazine),
VCR(Vincristine),
TMZ (Temozolomide),
CDDP(Cisplatin),
CBDCA(Carboplatin),
BBB: Blood Brain Barrier,
APL : acute promyelocytic leukemia

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