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Individuals' Leukocyte DNA Double-Strand Break Repair as an Indicator of Radiosurgery Responses for Cerebral Arteriovenous Malformations

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To evaluate the feasibility of using radiosensitivity of peripheral leukocytes as a predictor of clinical therapeutic responses to radiosurgery in individuals with cerebral arteriovenous malformation (AVM), we enrolled 18 patients years after they had received Gamma Knife radiosurgery for their cerebral AVM. The AVMs were shown with different degrees of regression in size in posttherapeutic periods. The peripheral leukocytes of these patients were collected at the last neuroimaging follow-ups. The leukocytes, before and 1 and 2 h after 8 Gy external gamma-irradiation, were evaluated for the amounts of DNA double-strand breaks (DSB) in 50 randomly selected individual nuclei by the neutral single cell gel electrophoresis, or so-called comet analysis. After being adjusted for gender and age at radiosurgery, the individuals with less posttherapeutic regression in AMV sizes or relatively poor or inadequate responses to radiosurgery were shown to have significantly higher DSB repair capacity on their leukocytes by comet analysis. These results suggested that *in vitro* radiosensitivity of peripheral leukocytes may provide valuable information for predicting therapeutic response or for adjusting irradiation doses in AVM radiosurgery.

INTRODUCTION

Radiosurgery has been used as an effective therapeutic alternative for cerebral arteriovenous malformations (AVM) for decades. In radiosurgery, the target dose is so high as to reach the therapeutic level and cause significant tissue damage while the dose to the surrounding brain tissue remains minimal. AVM radiosurgery using the Gamma Knife was reported to achieve 80–85% cure rates for small to medium-sized AVM in 2 to 3 years.[1,2] The therapeutic response seemingly stemmed from radiation damage to the endothelial cells of the AVM tissue, followed by a progressive thickening of the intimal layer and a proliferation of smooth-muscle cells. These cellular changes later developed into an extracellular matrix including type IV collagen and resulted in a narrowing of vascular lumen; they eventually led to vascular stenosis and a regression of the AVM nidus.[3] The contractile activity of myofibroblasts, the proliferation generated by irradiation, and the transformation of the resting cells into an activated form could be relevant to the regression process and eventual cure of AVM after radiosurgery.[4]

Multiple factors have been proposed to be responsible for successful AVM radiosurgery, including radiation dose at the margin of the target and grade and size of the AVM.[2,5–7] Furthermore, the angioarchitecture and hemodynamics of cerebral AVM also affected radiosurgical efficacy.[8] Technically, an insufficient coverage of AVM nidus within an effective isodose level or an inadequate length of follow-up period was assumed to be responsible for about 60% of therapeutic failures. Individual radioresistance over the internal vasculatures of AVM, however, was suspected to contribute to the other 40% of failures.[9] The current target dose selection in radiosurgery is based on diversified study reports published previously and the experience of the performers. The selection of irradiation dose has been based on a balance between therapeutic effects and adverse radiation effects. The target dose and treatment plan are usually considered to be optimized. However, unsuccessful treatments or unexpected severe adverse radiation effects may occur years after radiosurgery. Individual radiosensitivity is hypothesized to be one cause of

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result variance. If individual radiosensitivity can be evaluated before treatment, individualized treatment plans can be developed to have more effective treatments. Because the target area is inaccessible for *in vitro* observation before or after radiosurgery, little has been achieved to demonstrate possible local cellular radiosensitivity to radiosurgery in irradiated individuals.

Single cell gel electrophoresis, or comet assay, is a currently available molecular technique for evaluating cellular DNA double-strand breaks (DSB) after radiation.[10,11] It has also shown high sensitivity in measuring low levels of DSB for differentiating cellular radiosensitivity. We conducted the current study by applying comet assay to peripheral lymphocytes of AVM patients with the expectation of revealing the relationship between individual differences in radiosensitivity and therapeutic response. The study purpose was to evaluate the feasibility of applying radiosensitivity of peripheral leukocytes as a predictor of therapeutic responses in radiosurgery.

MATERIALS AND METHODS

Subject enrollment

From 1993 to 2002, a total of 399 patients received Gamma Knife radiosurgery for their cerebral AVM in our institute. Stereotactic magnetic resonance imaging (MRI) and X-ray angiography were employed for targeting the site of irradiation.[7] Clinically, we used MRI to regularly follow up on these patients in postradiosurgical years. If the AVM was seen totally regressed on MR images, X-ray angiography was subsequently performed to document the treatment result. The end point of their follow-up was a total regression of the AVM or when a second session of radiosurgery was performed on an incomplete regressed AVM at the end of the 3rd postradiosurgical year. Eighteen AVM patients who met one of the above criteria of follow-up were consecutively enrolled from 1999 to 2001. Their peripheral lymphocytes were collected for evaluating the postradiation DNA DSB and repair *in vitro*.

Blood collection and cellular processes

At the time of the follow-up on the X-ray angiography, 15 ml of blood was collected from each subject after informed consent. Postcentrifugation, the buffy coat leukocytes were isolated and maintained in RPMI 1640 culture medium mixed with 10% fetal calf serum and antibiotics.[12] Before and 1 and 2 h after 8 Gy γ-irradiation from a gamma-irradiator (dose rate 6 Gy/min) aerobically at room temperature, these leukocytes were kept at 37°C in a CO_2 incubator before being processed for single cell gel electrophoresis analysis (comet assay; Ma *et al.*, 1996). The cellular suspensions were each mixed with 1 ml of 1% low-melting agarose and gelled to frosted slides pretreated with 0.1% normal agarose. These slides were treated for 10 min in a pH 9.0 solution with 0.1 M EDTA and 2.5% SDS at room temperature to denature the cell membrane, nuclear membrane, and histone proteins surrounding the DNA double helixes. The slides sat submerged in running buffer on an electric field of 0.6 V/cm for 30 min to differentiate the migration of DNA molecules in the electric field. After rinse and stain in 1:100 SYBR Green I, each slide was coded, and 50 individual cells on the slides were randomly scored under a fluorescence microscope by one-side blindness. These cellular images were captured by a digital camera (Kodak DCS 315), analyzed by a self-designed image analysis program to estimate the relative lengths of nuclear "tails" from cellular nuclei, which represented the relative amounts of fragmented chromatin containing DNA DSB, and pulled to the cathode during previous electrophoresis.[11]

Indexes of radiosensitivity and therapeutic response

The leukocyte "repair capacity" for DNA DSB was defined as the tail-length difference between the first hour and the second hour posttreatment divided by the difference in the tail length of the first hour and the tail length before treatment, i.e., the decrease in the tail lengths (the relative amounts of DBS) during the first hour to the second hour of postirradiation, compared with the differences between those before irradiation to the first hour of postirradiation (Table 1). That is the repair ratio = (tail length $_{\text{Hour 1}}$ − tail length $_{\text{Hour 2}}$)/(tail length $_{\text{Hour 1}}$ − tail length $_{\text{Hour 0}}$), and the lower the repair ratio was, the more radiosensitive the cell was.

The therapeutic response (TR) of AVM radiosurgery was

Table 1. Summary of the demographic data and irradiation dosages of the 18 examined subjects in this study and AVM patient pool of the institute.

	Patient pool (N = 381)	Study subjects (N = 18)	*p* values
	Mean (SD)	Mean (SD)	Student's *t*-test
AVM volume (ml)	12.01 (12.27)	10.77 (7.86)	0.67
max	31.87 (3.89)	32.13 (2.89)	0.78
min	18.62 (2.52)	18.54 (2.36)	0.89
mean	25.25 (2.55)	25.33 (2.07)	0.89
Age (yr)	30.54 (14.55)	28.67 (11.62)	0.59
	chi square test		
Male/Female Ratio	(214/167) = 1.28	(10/8) = 1.25)	0.96

Table 2. Characteristics of arteriovenous malformation before and after radiosurgery correlated with the DNA DSB repair capacity in peripheral lymphocytes after 8 Gy γ-irradiation *in vitro*.

No.	Gender/ Age	AVM size GKRS[a] (ml)		Target dose of GKRS[a] (Gy)			Duration of follow-up (months)	DNA repair capacity *in vitro* and therapeutic response			
		Before GKRS[a]	After GKRS[a]	Minimum	Maximum	Average		RR[b]	TR_{max}[c]	TR_{min}[d]	TR_{ave}[e]
1	M/ 25	13.9	3.6	20.0	40.0	NA*	40	14.0	4.63	9.26	—
2	M/ 15	8.9	1.3	18.0	36.0	NA*	45	5.0	5.27	10.54	—
3	M/ 30	18.7	39.7	17.0	34.0	19.8	53	35.0	–6.20	–12.40	–10.65
4	F/ 9	0.3	0	21.0	28.0	23.3	18	0.0	19.84	26.46	23.84
5	F/ 21	6.4	0	17.6	32.0	23.9	39	20.0	8.01	14.57	10.73
6	M/ 33	12.3	0	17.5	30.0	23.6	25	–6.0	13.33	22.86	16.95
7	M/ 33	26.0	0	17.0	31.0	23.1	33	8.7	9.78	17.83	13.12
8	M/ 46	10.9	0	16.5	33.0	24.0	38	11.5	7.97	15.95	10.96
9	M/ 38	23.5	0	16.5	33.0	22.5	39	3.5	7.77	15.54	11.40
10	F/ 26	4.7	0	18.0	30.0	24.9	22	49.7	15.15	25.25	18.25
11	F/ 21	13.0	32.6	17.6	32.0	22.7	62	47.0	–7.60	–13.82	–10.71
12	F/ 13	0.9	0.8	23.0	32.0	29.8	39	60.5	0.58	0.80	0.62
13	F/ 48	1.0	0.2	25.0	35.7	30	43	24.7	5.28	7.53	6.28
14	F/ 20	0.4	0	19.5	30.0	24.9	24	12.2	13.89	21.37	16.73
15	M/ 39	18.3	0	16.5	30.0	22.2	21	1.7	15.87	28.86	21.45
16	M/ 26	15.6	0	17.0	30.1	23.4	25	6.1	13.29	23.53	17.09
17	F/ 47	13.0	1.1	18.0	29.5	23.5	17	79.6	18.27	29.94	22.93
18	M/ 26	6.1	0	18.0	32.0	24.3	18	–0.4	17.36	30.86	22.86

[a]GKRS: Gamma Knife Radiosurgery

[b] $$\text{Repair Ratio (RR) of lymphocytes} = \frac{\text{tail length}_{\text{hour 1}} - \text{tail length}_{\text{hour 2}}}{\text{tail length}_{\text{hour 1}} - \text{tail length}_{\text{hour 0}}} \times 100\%$$

[c] $$\text{Therapeutic Response}_{\max}\ (TR_{\max}) = \frac{\text{AVM size}_{\text{Last imaging follow-up}} - \text{AVM size}_{\text{Before radiosurgery}}}{(\text{follow-up duration in months}) \times (\text{estimated maximum irradiation doses on AVM nidus})} \times 100\%$$

[d] $$\text{Therapeutic Response}_{\min}\ (TR_{\min}) = \frac{\text{AVM size}_{\text{Last imaging follow-up}} - \text{AVM size}_{\text{Before radiosurgery}}}{(\text{follow-up duration in months}) \times (\text{estimated minimum irradiation doses on AVM nidus})} \times 100\%$$

[e] $$\text{Therapeutic Response}_{\text{ave}}\ (TR_{\text{ave}}) = \frac{\text{AVM size}_{\text{Last imaging follow-up}} - \text{AVM size}_{\text{Before radiosurgery}}}{(\text{follow-up duration in months}) \times (\text{estimated average irradiation doses on AVM nidus})} \times 100\%$$

*The two patients were treated at the first stage of the radiosurgery program of the institute. An old version of the dose-planning system was used at that time. It was not possible to define the average dose in this system.

defined as the ($AVM\ size_{\text{before radiosurgery}} - AVM\ size_{\text{last imaging follow-up}}$) × 100%/[(follow-up duration in months) × (estimated maximum, prescribed, or average irradiation doses on AVM nidus)]. The maximum irradiation dose was the highest dose prescribed to the AVM, the prescribed dose indicated the isodose level that covered the margin of AVM nidus, and the average dose was the average irradiation dose to the target volume derived from the dose volume histogram. The AVM volume before radiosurgery was defined as the so-called best-fit isodose volume, which was the volume of tissue within the prescribed dose level.[6] At the last postradiosurgical follow-up, AVM volume was defined as zero if AVM was demonstrated by X-ray angiography to be cured. For AVM not yet cured at the last imaging follow-up, the remaining AVM volume was defined by the sum of all nidus slabs delineated on T1-weighted MR images.[13]

Based on the estimated maximum, prescribed, and average irradiation doses and on the AVM volumes before radiosurgery and at the last postradiosurgical follow-up, TR was coined as TR_{max}, TR_{min}, and TR_{ave}, respectively.

Statistical analysis

The Student's *t*-test and the chi-square test were employed to compare the differences in characteristics of the total pool of 381 patients and these 18 study subjects. The multiple linear regression analysis was employed, and the ages at the radiosurgery, gender, and cellular repair capacity at this evaluation were considered and analyzed by the GEE in SAS (version 6.12; SAS Institute Inc., Cary, NC, USA, 1989). A test was considered statistically significant if its *p* value was less than 0.05 and borderline significant if the *p* value was from 0.1 to 0.05.

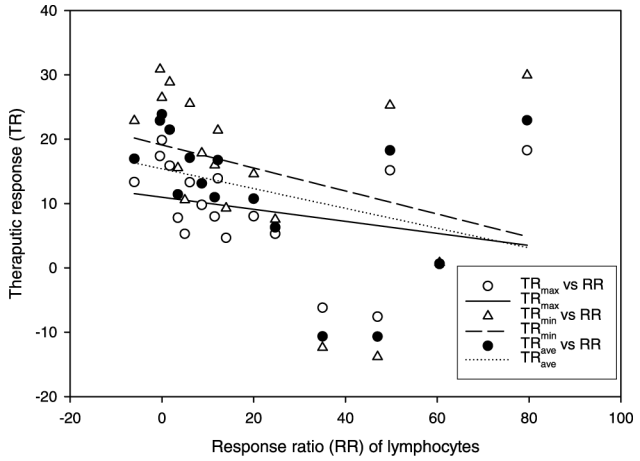

Fig. 1. The correlation between therapeutic responses (TR) in TR_{max}, Tr_{ave}, and TR_{min} and repair ratios (RR) of the lymphocytes.

RESULTS

The demographic distribution of these 18 patients and irradiation dosages were similar to the rest of patient pool of 381, shown in Table 1.

The demographic data of AVM in these 18 subjects are demonstrated in Table 2. These data included before and after radiosurgery and the DNA double-strand repair capacity *in vitro* in their leukocytes; age; the target dose of radiosurgery in maximum, prescribed, and average (Gy); and the duration of follow-up (months). The correlation between therapeutic responses (TR) and repair ratios of the lymphocytes are shown in Fig. 1.

As a result of multiple linear regression analysis, which considered patient genders and ages at radiosurgery, the therapeutic response (TR) of radiosurgery is shown in Table 3 to have statistical or borderline significant negative association with the "repair capacity" of the peripheral leukocytes after 8 Gy γ-irradiation *in vitro* in this small number of subjects. [The parameter estimates for the repair ratios of TR_{max}, TR_{min}, and TR_{ave} were -0.19 ± 0.09, -0.30 ± 0.15, and -0.25 ± 0.13, and the p values were 0.04, 0.055, and 0.055, respectively.] On the other hand, the ages and genders of these patients were not associated with the therapeutic response. The correlation between therapeutic responses (TR) in TRmax, Trave, and TRmin and repair ratios (RR) of the lymphocytes are shown in Fig. 1.

DISCUSSION

The results indicated that subjects with better therapeutic response from AVM radiosurgery; i.e., those having a larger reduction in the volumes of AVM nidus after radiosurgery, were shown to have significantly less DSB repair capacity in their leukocytes toward external γ-irradiation. Because the DNA DSB generated from external irradiation were mostly similar in amounts at the first initial damage, the different amounts of DSB remaining in the nuclei 1 or 2 h postirradiation were shown to reflect individual cellular repair capacity toward DSB. Individuals with leukocytes showing less remaining DSB at the second hour postirradiation in the above analysis were assumed to have better repair capacity or more radioresistance over their endothelial cells of the AVM nidus. From previous histopathological studies,[3,4] AVM patients with better therapeutic response after radiosurgery were hypothesized to have more endothelial damage and less DNA repair. These patients might have carried large extents of extracellular matrix expansion and consequently a higher tendency toward total obliteration of the AVM nidus after irradiation. This information provided the important and unique implication that AVM patients who demonstrated relative radioresistance in their leukocytes and larger repair ratios after *in vitro* external radiation might require dose modification for radiosurgery in the future.

The associations between radiosensitivity of lymphocytes and endothelial cells have been suggested in previous studies. The ability of normal blood-borne lymphocytes to enter peripheral lymph nodes was markedly depressed in mice that had been exposed to 7.5 Gy of gamma-irradiation.[14] Preactivated peripheral blood mononuclear cells caused endothelial cell (EC) damage and may prevent blood vessel repair by arresting the proliferation of ECs.[15] This radiation-induced effect was observed to last longer than 6 months after irradiation, and its magnitude was observed to be radiation-dose dependent. On the other hand, it was suggested that irradiation-induced endothelial cell edema and microvascular occlusions occurred within lymphocyte-receptive areas of the nodal microvasculature.[16] Specific helper T-cell functions, such as contact hypersensitivity and alloantigenic responses, remained significantly depressed in the lymph nodes of irradiated mice for prolonged periods (up to 60 weeks).[17] These were further evidenced in radiation-induced damage to high endothelial venules by interfering with the normal passage of lymphocytes from the blood into lymph nodes via a specific interaction between lymphocytes and high endothelial venules.[18] Gamma-irradiation on human T lymphocytes was also observed to impair the ability of lymphocytes to adhere to both fibroblasts and endothelial cells. The impairment of cellular immune function after irradiation in vivo was addressed to be caused, in part, by defective T lymphocyte emigration and localization at inflammatory sites.[19]

The response of AVM-relevant vasculatures to irradiation is a multifactorial, multiphasic process. Factors such as smaller AVM volumes, number of draining veins, patients at young ages, and hemispheric AVM locations were shown to be significantly associated with the clinical outcomes in a series of 220 patients.[20] In one of our previous studies, we found that AVM volume was the most decisive factor for a successful radiosurgery.[7] In another study with retrospective analysis on 1,006 AVM patients treated by Gamma Knife

Table 3. The association between therapeutic responses (TR) of the AVM radiosurgery and the associated variables by the multiple linear regression analysis.

Model: TR_{max} = a + b*age + c*gender + d* repair ratio

Parameter		Estimate	Standard Error	Wald 95% Confidence Limits		*P* value
Intercept	a	11.49	4.95	1.79	21.19	0.02**
Age	b	0.18	0.15	–0.12	0.48	0.25
Gender	c	–6.64	4.41	–15.29	2.002	0.13
Repair ratio	d	–0.19	0.09	–0.37	–0.009	0.04**

Model: TR_{min} = a + b*age + c*gender + d* repair ratio

Parameter		Estimate	Standard Error	Wald 95% Confidence Limits		*P* value
Intercept	a	16.16	8.36	–0.225	2.55	0.053
Age	b	0.34	0.26	–0.170	0.85	0.19
Gender	c	–8.12	7.45	–22.72	6.49	0.28
Repair ratio	d	–0.30	0.15	–0.599	0.01	0.055*

Model: TR_{ave} = a + b*age + c*gender + d* repair ratio

Parameter		Estimate	Standard Error	Wald 95% Confidence Limits		*P* value
Intercept	a	16.16	8.36	–0.225	32.55	0.053
Age	b	0.34	0.26	–0.170	0.85	0.19
Gender	c	–8.12	7.45	–22.72	6.49	0.28
Repair ratio	d	–0.30	0.15	–0.599	0.01	0.055*

*$0.05 < p < 0.1$; **$p < 0.05$.

radiosurgery, however, it was found that the probability of AVM cure was related to the prescribed target dose only.[21] In the same study, the AVM volume was not shown to affect the radiosurgical response, but centrally located AVM tended to have a better chance of being cured than hemispheric ones.[22] Another serial analysis on the in-field obliteration in 197 AVM patients with 3 years of angiographic follow-up revealed a significant independent correlation with a prescribed target dose, though not with volume or a maximum dose.[23] But the diversified study reports have been used as the guideline of dose selection in AVM radiosurgery among institutes worldwide, all radiosurgical treatments have been considered to be optimal ones. The knowledge of dose selection and effect of demographic data on therapeutic response reported in the literature were therefore merged into the current clinical practice of AVM radiosurgery. In the current study, the analysis was based on the assumption that the irradiation dose for these 18 AVM patients had been ideally selected by a consideration of both therapeutic response and risk of complication. Under the presumably selected condition, we hypothesized that the individual differences in radiosensitivity might play a role in the therapeutic response of AVM radiosurgery.

Gamma Knife radiosurgery employs cross-fired focused irradiation and takes two to three years to yield a final therapeutic result. Whether the blood samples were taken before or after radiosurgery treatment should have made no difference in the analysis of the comet assay of individual radiosensitivity. In this study, the blood samples were taken after treatments because of the design of the study.

Radiosurgery has been shown to be an effective and safe alternative for children with cerebral AVM.[24] The high cure rates (65% to 80%) and low morbidity incidence suggested the primary role of radiosurgery for pediatric AVM. In a review of 58 reported pediatric AVM cases, however, it was postulated that an increase in the size of an untreated AVM occurred particularly in childhood.[25] A small possibility was found that the postradiosurgical AVM may reappear years after it was noted to be cured, especially in pediatric patients.[25] The repair capacity of AVM vasculatures in AVM seemed to be higher in childhood compared to adults, though the difference might be minor. In the current study, however, the ages of patients in radiosurgery were not shown to be related to therapeutic response, but this might be due to small numbers of study patients in different age distributions.

The effect of this current study should be, first, that the individual radiosensitivity may be involved in the different therapeutic response seen in AVM radiosurgery. Second, single cell gel electrophoresis after external irradiation or a so-called comet analysis of peripheral leukocytes may serve as a tool to evaluate radiosensitivity for AVM patients via cellular DNA strand breaks (DSB) and repair. Third, a preradiosurgi-

cal comet analysis of AVM patients may be helpful for optimizing therapeutic strategy in AVM radiosurgery.

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