Combination Effects of Pulsed Magnetic Stimulation (MS) and Imatinib Mesylate (IM) on IM-resistant Leukemic Cells

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Abstract

Effects of pulsed magnetic stimulation (MS) on human breakpoint cluster region-abelson *(BCR/ABL*) (+) leukemic cells were examined. MS conditions were as follows: 0.25 T, 25 pulses/sec, and 1000 pulses/day/sample. Combinational use of MS and the anti-cancer agent, imatinib mesylate (IM, Gleevec®), induced sufficient cell death in IM-resistant *BCR/ABL* (+) cells. This effect was due to the activated apoptosis mediated by the mitochondorial dysfunction and enhanced intracellular release of cytochrome c. These results suggest that combinational use of MS and IM has a therapeutic potential against IM-resistant *BCR/ABL* (+) leukemia by enhancing IM efficacy via mitochondorial dysfunction.

1. Introduction

The breakpoint cluster region-abelson (*BCR/ABL*) is a chimeric gene, generated from the Philadelphia (Ph) chromosome translocation, t(9;22)(q34;q11), in human leukemic cells [1]. This gene encodes a constitutively active cytoplasmic tyrosine kinase, which is critical for transformation of hematopoietic cells [1]. Regarding *BCR/ABL* (+) leukemia, such as chronic myelogeneous leukemia (CML), imatinib mesylate (IM, Gleevec®) was developed as a potent and specific inhibitor of ABL tyrosine kinase [2]. However, acquired resistance to IM has recently developed in a substantial fraction of patients [3].

The application of bioelectric and bioelectromagnetic methods for cancer research and therapy has increased drastically, sometimes in combination with specific anticancer agents such as bleomycin or actinomycin [4]. Pulsed magnetic stimulation (MS) is the use of pulsed magnetic fields to induce electric fields in tissues by electromagnetic induction without the need for surgery or external electrodes [5]. In a previous report, our group demonstrated the enhanced IM efficiency in human *BCR/ABL* (+) CML cells by combinational use of MS (0.25 T, 25 pulses/sec, and 1000 pulses/day) and IM, and suggested that MS would be useful for cancer treatment [6]. However, the effects on IM-resistant cells and the detail of the mechanisms have not been thoroughly investigated.

In this study, we examined the effects of MS on IM-resistant *BCR/ABL* (+) cells. The mechanisms of the combination effect by MS exposure were also investigated.

2. Materials and Methods

Cell line

TCC-Y/sr (TCC-Y/T315I) cells, IM-resistant *BCR/ABL* (+) leukemic cells, were derived from patients. The IC_{50} IM of TCC-Y/sr cells was 22.47 ± 3.57 μM while the IC_{50} IM of IM-sensitive *BCR/ABL* (+) cells, K562 and TCC-S, was 0.16 ± 0.05 μM and 0.18 ± 0.04 μM, respectively. The TCC-Y/sr cells were cultured and maintained in RPMI-1640 medium (Sigma, St. Louis, USA) containing 10% fetal bovine serum (FBS, Sigma) and 1% antibiotic-antimycotic solution (Sigma). Cells were cultured in annular dishes (inner diameter = 30 mm, outer diameter = 54 mm, thickness = 10 mm: Falcon, Franklin Lakes, USA) to exclude non-magnetic effects.

Exposure systems and stimulus conditions

MS was performed with a magnetic stimulator (Nihon Kohden Co., Tokyo, Japan), which delivered biphasic cosine current pulses for 238 μs. A circular coil (inner diameter = 15 mm, outer diameter = 75 mm) was used in the experiments. Stimulus parameters were determined based on our previous studies [6]. The MS conditions were as follows: peak magnetic field = 0.25 T, frequency = 25 pulses/sec, and 1,000 pulses/sample/day. Figure 1C shows the stimulus pattern in one day. The dish was placed 5 mm above the stimulus coil and exposed to MS (Fig. 1A). Cells were exposed to MS for three days every 24 hrs from the start of the drug treatment.

The distributions of the magnetic fluxes and eddy currents in MS were calculated using a computer program based on the finite element method [7]. The magnetic flux and magnetically induced eddy current were estimated in the range of 0.11 - 0.18 T and 26.8 - 38.1 A/m^2, respectively, in the dish (Fig. 1B).

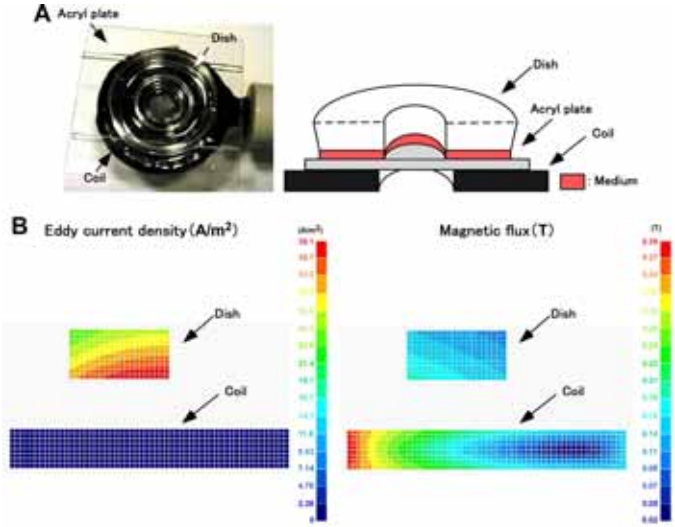

Fig. 1 A: Exposure system B: Distribution of eddy current density and magnetic flux

Cell viability assay of TCC-Y/sr cells

The IM was obtained from Sequoia Research Products (Pangbourne, UK). The TCC-Y/sr cells were seeded on a dish at a density of 3.0×10^5 cells in a 3-ml medium and incubated for 1 hr before MS exposure. Viable cells were determined using the WST-8 assay kit (Tetra Color ONE, Seikagaku Corporation, Tokyo, Japan). Cells were transferred from each dish to a 96-well dish and 10 μl of Tetra Color ONE solution were added to each well. After 3 hrs of incubation, the absorbance of a 450-nm wavelength (630-nm as a reference) was detected using a spectrometer. Data measurement was repeated three times for one sample and then averaged. For data analysis, the Student *t*-test was performed. The probability (p) values < 0.05 were considered to be statistically significant.

Flow cytometric analysis

Apoptotic cells were assessed using the Annexin-V-FLUOS Staining kit (Roche diagnosis, Nutley, USA). Mitochondorial transmembrane potential (ΔΨm) was detected using the MitoProbe DiOC2(3) Assay kit (Invitrogen Japan, Tokyo, Japan). Cytosolic release of cytochrome c was detected using the InnoCyte™ Flow Cytometric Cytochrome c Release Kit (Calbiochem, Darmstadt, Germany). After MS, we analyzed the cells with the flow cytometer (Epics XL ADC, Bechman coulter, Fulerton, USA) and processed the data with the analysis program (Expo32).

3. Results

Cell viability assay of TCC-Y/sr cells

Table 1 shows the effect of MS on cell viability at 72 hrs from drug treatment. Significant decreases in cell viability were observed in both IM 20-μM + MS and IM 40-μM + MS treated cells while untreated or IM alone showed insufficient effects. MS alone had little effect on cell viability.

Second, to clarify the timecourse of the combination effects, TCC-Y/sr cells were treated by IM 40 μM alone or with MS. Cell viability was then examined. Figure 3 shows the timecourse of the combination effects. The combination effects were observed from 24 hrs from drug treatment compared with IM alone. Relative values of IM alone-treated cells were 13.7 ± 0.0% (at 24 hrs), 13.7 ± 0.0% (at 48 hrs), and 13.7 ± 0.0% (at 72 hrs), respectively.

	% Control
Control	100.0±0.0
MS alone	95.2±0.1
IM 20 μM	97.6±0.0
IM 20 μM+MS	62.5±0.1*
IM 40 μM	37.7±0.0***
IM 40 μM+MS	13.7±0.0***††

Table 1 Effects of MS on TCC-Y/sr cell growth. Results were expressed as Mean±S.E. * : p<0.05 v.s. control, IM 20 μM. *** : p<0.001 v.s. control †† : p<0.01 v.s. IM 40 μM.

Flow cytometric analysis

As shown in Fig. 2A, the number of early apoptotic cells (annexin V(+) / PI (-)) increased in IM 40 μM + MS at 72 hrs from the drug treatment. Additionally, necrotic cells (annexin V(+) / PI (+)) also increased by the combination treatment.

Then, since the loss of ΔΨm represents mitochondorial dysfunction, and IM treatment induces cell death via the mitochondorial pathway [8], the changes in ΔΨm were examined. As shown in Fig. 2B, after treatment with IM 40 μM + MS, ΔΨm decreased dramatically while IM 40 μM alone and control cells showed no effect.

Figure 2C shows the cytosolic release of cytochrome c after IM 40 μM or IM + MS. The IM + MS treatment caused a decrease in fluorescence intensity, suggesting that IM + MS enhanced cytosolic release in cytochrome c.

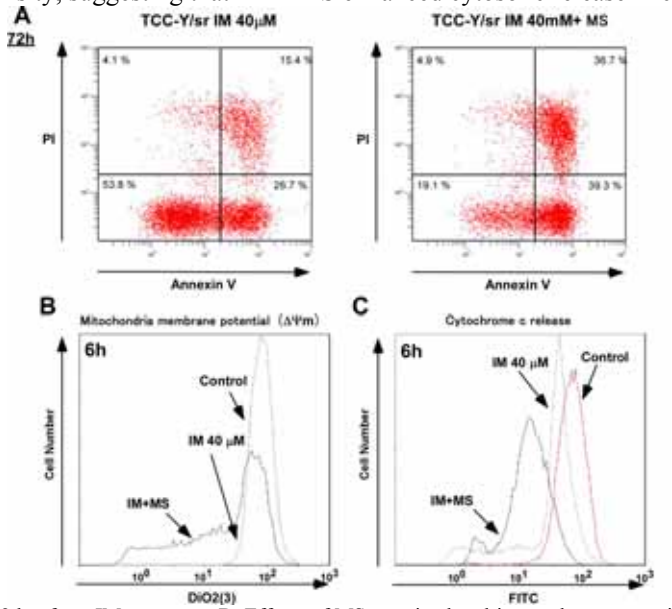

Fig. 2 A: Annexin / PI staining at 72 hrs from IM treatment. B: Effects of MS on mitochondria membrane potential. C: Effects of MS on intracellular release of Cytochrome c

4. Discussion

The T315I mutation causes the drastic conformational changes in the *BCR/ABL*-ATP binding region; therefore, it has been reported as the strongest IM-resistance [8]. In spite of the success in overcoming IM-resistance by the second generation of ABL kinase inhibitors (e.g., Dasatinib and Nirotinib), IM-resistance due to T315I mutation has not been completely solved [3]. Our group examined the effects of MS on IM-resistant *BCR/ABL* (+) leukemic cells baring T315I mutations (Table 1 and Fig. 3). As shown in Table 1, IM + MS induced sufficient cell growth inhibition compared with the control, MS alone, and IM alone. The timecource of the combination effects indicates that the growth inhibition effects occurred much earlier in IM + MS compared with IM alone (Fig. 3). These results indicate that

the combinational use of IM and MS is necessary for sufficient cell growth inhibition in IM-resistant cells, and they also suggest that changes in cellular functions occur at the earlier time point with the combination treatment.

Since IM causes the cell cycle delay in *BCR/ABL* (+) cells [8], we performed Annexin V / PI staining to clarify whether or not the decrease in cell viability came from the enhancement of cell death. As shown in Fig. 4, apoptotic cells (Annexin (+) / PI (-)) and necrotic cells (Annexin (+) / PI (+)) increased in IM + MS at 72 hrs from the drug treatment.

Then, changes in the mitochondrial $\Delta\Psi m$ and the cytosolic release of cytochrome c were examined based on former reports on the IM-induced cell death pathway [8]. The early change in mitochondrial $\Delta\Psi m$ was observed in IM + MS at 6 hrs after drug treatment while IM or MS alone showed little change (Fig. 5). Additionally, increased cytosolic release of cytochrome c occurred only in IM + MS cells (Fig. 6). These results demonstrate that the early changes in mitochondrial functions, including cytosolic release of cytochrome c, were caused by IM + MS specifically, and suggest that these changes led to the drastic increase in dead cells.

5. Conclusion

In conclusion, we have presented that the combination effects of IM and MS induced sufficient cell death in IM-resistant *BCR/ABL* (+) cells. This effect was due to the activated apoptosis mediated by the mitochondorial dysfunction, and it enhanced intracellular release of cytochrome c. These results suggest that combinational use of MS and IM has a therapeutic potential against the IM-resistant *BCR/ABL* (+) leukemia by enhancing IM efficacy via mitochondorial dysfunction.

6. Acknowledgments

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7. References

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