Report on "Radiation Disaster Recovery Studies"

Course: Radiation Disaster Medicine
Name: Nguyen Quang Tam

- **Regarding "Radiation Disaster Recovery Studies"**

Phoenix Leader Education program was initiated by Hiroshima University in 2011 for renaissance from radiation disaster and supported by MEXT (Ministry of education, Culture, Sports, Science and Technology of Japan), right after the severe earthquake caused the second worst radiation accident in the world at Fukushima Dai-ichi nuclear power plant, since Chernobyl radiation disaster (Soviet Union, 1986). [(1)]

Japan has suffered during several nuclear disasters such as Hiroshima and Nagasaki atomic bomb (August, 1945), Tokai-mura nuclear accident (1997, 1999) and recently was Fukushima nuclear accidents (March, 2011). Those accidents gave large number of damage and experiences for Japanese government and scientists in facing to serious problems of human health, environment and social recovery. After Fukushima accident, Hiroshima University has realized that there is needing of a well-organized education program to foster students in major fields that can have enough combined knowledge and interdisciplinary skills in radiation disaster science. That gave me a chance to study in Radiation disaster medicine course of the program beside my major in oral health sciences.

During the whole program, I have good chances to learn from the basic knowledge such as history and impact of nuclear accidents on human health in molecular biology and disease pathology to advanced course in radiation oncology and head and neck cancer treatment. In addition, combination with radiation environment subjects helped me to understand clearly about risk of radioactive sources in our disaster area and its long-term exposure. Together with direct radiation-induced damage in human health, this is the first time I can learn about physiological effect that evacuees are having, which can indirectly lead to changes in their glucose metabolism, causing diabetes [Satoh et al. (2015)].

Beside many useful lectures in school from global experts, I have had many opportunities to join several common field trips and short field trips in Tokyo, Ibaraki (Chiyoda factory), Fukushima and many symposiums in radiation physics and health management in Nagasaki, Tokyo Institute of Technology, Fukushima, Seoul (ICRP 2015), United States of America (Colorado State University, 2016) and especially the long-term internship in International Atomic Energy Agency (IAEA, 2016) to study about clinical applications of mesenchymal stem cells in treating radiation-induced lesions.

Radiation has various negative effects on human health such as cell death in molecular level and organ damage and mortality risk to human when exposed to high radiation dose. It also increased the risk of having cancer when individual exposed with radiation over 1Gy while

radiation effect of under 100mGy is uncertain [Kamiya et al. (2015)]. After Fukushima accident, residents and evacuees were at risk at radiation exposure and changing of their life, giving many challenges to government and scientists to protect their people and to recovery process [Hasegawa et al. (2015); Ohtsuru et al. (2015)].

Being a graduate student in both Phoenix program and Department of Molecular Oral Medicine and Maxillofacial surgery gave me a fundamental knowledge in oral cancer, effect of radiation and radio-chemotherapy for treating cancer. With lack of knowledge about effect of low dose radiation on human health and also in cancer treatment which I have learned during four years in Phoenix program gave me an idea to study effect of radiation on squamous cell carcinoma, especially with low dose radiation.

References:

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- **Title of Doctoral Thesis:**

Establishment and characterization of radiation resistant strains from squamous cell carcinoma cell lines in serum-free defined culture

- **Summary of Doctoral Thesis:**

Introduction

Squamous cell carcinoma (SCC), including oral squamous cell carcinoma (OSCC), has been increasing in the world and being the most common cancer in South-East Asian countries where have a betel-quid and areca-nut chewing habit. It has been considered that cancer cells are functionally heterogeneous that undergo not only proliferation but also

differentiation and maturation to a certain degree and contain a small population of cancer stem cells (CSCs). It seems logical that cures of cancer can be achieved only if the CSCs population is eliminated.

On the other hand, there are growing evidences that CSCs are genetically radio-resistant (RR) and might be resistant to other cancer therapies. Therefore, to clarify biological effects of radiation on SCC cell lines *in vitro* and *in vivo* might be worthwhile to circumvent radiation resistance for oral cancer. Currently, there are no report of establishment of RR-SCC cells in serum-free defined culture, which ensures exact biological mechanisms and prevent instability of using serum.

In this study, first I have tried to isolate and establish RR-SCC strains from SCC and OSCC cell lines, and then characterized their cellular and molecular properties in serum-free defined culture.

Materials and methods

Two SCC cell lines were used in this study including A431 derived from vulvar SCC, and NA/HO-1-N-1 from OSCC. The cells were cultured in serum-free DF6F medium (1:1 mixture of DMEM and Ham-F12 medium supplemented with insulin (10μg/ml), transferrin (5μg/ml), 2-aminoethanol (10μM), sodium selenite (10nM), 2-mercaptoethanol (10μM), and oleic acid conjugated with fatty acid-free bovine serum albumin (9.4μg/ml)).

All cell lines were irradiated weekly at a dose of 2.2Gy/day, 4days/week with a low dose rate (LDR) irradiation system (RM1000, Chugai Technos, Japan), or at 5Gy/5.75min, twice a week with a high dose rate (HDR) system (Gamma cell 40 Exactor, Best Theratronics, Canada) in serum-free defined culture. After irradiating 60Gy as a whole dose by LDR and HDR irradiation system, we have isolated 4 RR sub-strains from 2 SCC cell lines. These radiation resistant (RR) strains were continuously irradiated every week at a dose of 0.5Gy from passage 5. To confirm the radiation resistance of these sub-strains, we have performed a clonogenic survival assay as follow. The parental and RR strains were irradiated at a dose of 0Gy, 2Gy, 4Gy, 6Gy, and 8Gy, respectively, and examined radiation resistance by clonogenic survival assay. After 14 days of culture, the colonies were stained with Giemsa, and counted.

To clarify the biological properties of these cells, several cellular abilities such as growth in monolayer culture, sphere formation in suspension culture, and migration in Boyden-chamber method were examined. For the growth assay, the cells were seeded at 10^4cells/well in 24-well plate and counted cell numbers every day by the Coulter Counter. For sphere formation assay, the cells were seeded in 35mm prime surface (low-attachment) dish in DF6F serum-free medium at 10^3cells/dish, and then sphere numbers were counted on day 5. For migration assay, the cells were seeded at $5x10^4$cells/well in 24-well collagen coated chemotaxicell well in DF medium supplemented with 0.1% BSA and stained with Giemsa for counting number of migrated cells/mm^2. The ratio of CD133 positive cell in each cell line was examined by flowcytometry as cancer stem cell marker.

Total RNAs extracted and purified from all cell lines, were used for Real Time-quantitative PCR (RT-qPCR), and DNA microarray analysis.

To study the tumor forming ability in nude mouse, parental and RR cells (0.25 $x10^6$ - $1x10^6$ cells/100μl DF) were injected subcutaneously to the dorsal back skin of nude mice (BALB/c-nu/nu), and tumor size was measured every week. Then the tumors were excised, weighted, fixed in 4% paraformaldehyde for 24hr, and embedded in paraffin for H&E and immuno-histochemical staining.

To investigate which gene has important role in radiation resistance of those cancer cells, several high expressed genes in DNA micro array data were picked up for clonogenic survival assay after cancer cells were transduced with selected siRNA. Relation of these genes to pluripotent stem cells markers was verified by RT-qPCR after silencing with siRNA.

Results

By using LDR and HDR system, A431-LDR, NA-LDR, A431-HDR, and NA-HDR were radiation resistance cell lines and successfully isolated in serum-free defined culture. The D_{37} value of A431-LDR, A431-HDR, parental A431, NA-LDR, NA-HDR, and parental NA was 5Gy, 3.7Gy, 2.3Gy, 7.5Gy, 5.5Gy and 4.6Gy, respectively. These cells exhibited higher expression of cancer stem cell marker such as CD133, higher sphere formation and higher migration abilities compare to those of parental cell lines. Expression of pluripotent stem cell markers such as Nanog, Sox2, and Oct4 were not consistent among the RR cells. As a result of DNA microarray analysis, the RR cell lines showed elevated expression of KRT13 (200 folds), insulin-growth factor 2 (IGF2), chemokines (IL1R2, CCL20) and genes involved in tumor micro environment (S100A) and metastasis (MMPs). In addition, the RR cells exhibited higher tumor forming ability compared to parental cells in nude mice xenograft. In addition, two genes were confirmed to have effects in this cancer cell radiation resistance.

Discussion

Radiation resistant strains from SCC cell lines were successfully isolated in serum-free defined culture. These cells exhibited CSCs-like properties such as high stem cell marker expression, sphere forming ability, migration activity *in vitro* and a high tumor forming ability *in vivo*.

These radiation resistant cells might be very useful not only to study cancer stem cells but also to circumvent radiation resistance for the novel cancer treatment modality.