The Suppression of Hypoxia-inducible Factor and Vascular Endothelial Growth Factor by siRNA Does not Affect the Radiation Sensitivity of Multicellular Tumor Spheroids

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siRNA/HIF/VEGF/Spheroids/Hypoxia.

Background: The hypoxic microenvironment is closely associated with the radiation resistance of tumor cells. Hypoxia induces several genes such as hypoxia-inducible factor (HIF-1) and vascular endothelial growth factor (VEGF) to promote tumor cell growth and survival. The up-regulated expression levels of HIF-1 and VEGF in tumor cells also correlate with their resistance to radiation, suggesting that these genes are potential therapeutic targets for strategies designed to enhance radiation effects. To further investigate this possibility, we investigated the effects of suppressing these genes upon the radiation sensitivity of cancer cells. We conducted these experiments using multicellular spheroids as a three-dimensional in vitro tumor model and RNA interference as the method of gene suppression. Material and methods: SQ5 human lung carcinoma cells were treated with HIF-1/VEGF siRNA and/or radiation. Reversed transfection methods were employed for the spheroids. Gene expression was analyzed using quantitative RT-PCR and western blotting. Cell toxicity was qualified by colony formation assay. Results: Compared with monolayer cells, spheroids showed up-regulated expression of HIF-1 and increased radiation resistance. Hypoxic conditions elevated the expression of HIF-1 and VEGF and enhanced the surviving fraction of spheroids after exposure to radiation. However, when the expression of HIF-1 and VEGF was down-regulated by transfection of targeting siRNA, this did not influence the cytotoxic effects of the radiation under either normoxic or hypoxic conditions. Conclusions: We have established a method to transfect siRNA into spheroid cells. Our current data indicate that the functions of HIF-1 or VEGF are independent of radiation sensitivity in spheroids under either normoxic or hypoxic conditions.

INTRODUCTION

Although radiation therapy is one of the most effective current treatments for cancer its effects remain quite variable among different types of tumors. Tumor recurrence following a course of radiation therapy results from radiation resistance in some cells which can survive and re-proliferate. It is well known that one of the major reasons for the failure of radiation therapy is hypoxia.1-3 The development of a hypoxic microenvironment induces the expression of a variety of genes in tumor tissue that act to facilitate the supply of oxygen and nutrients and to promote tumor cell growth and survival. One such gene is hypoxia-inducible factor (HIF-1).1,3-5 HIF-1 is not detected under normoxic conditions but is expressed and stabilized as an initial response to hypoxia and then accumulates in the nucleus. Hence, HIF-1 is suitable indicator of cells growing under hypoxic conditions.

During the response to hypoxia in cells, HIF-1 plays an important mediating role via the control of its downstream genes. For example, HIF-1 induces angiogenic factors such as the members of the vascular endothelial growth factor (VEGF) families.6,7 VEGF directly facilitates tumor angiogenesis thus accelerating tumor growth and several studies have demonstrated that high HIF-1 and VEGF expression levels in tumor cells correlate with a greater malignant potential, more aggressive growth characteristics,
and often resistance to radiation.\textsuperscript{1,4–6} According to clinical studies, up-regulated HIF-1 and VEGF levels in tumor cells are associated with tumor resistance to radiotherapy and a poor prognosis.\textsuperscript{8–11} The mechanisms by which HIF-1 and/or VEGF are involved in radiation resistance are unclear however and remain under investigation.

Spheroids are three dimensional tumor cell aggregations that can be used as an experimental solid tumor model and provide a good \textit{in vitro} system to mimic the features of solid tumors.\textsuperscript{12–14} They have therefore been utilized in a broad spectrum of studies in cancer biology. These aggregations can be characterized by tumor-like structures of a high cell-density, which severely limits the diffusion of molecules and oxygen. Hence, spheroids bear the hallmarks of the heterogeneous cell subpopulations that are found in solid tumors i.e. an outer layer comprising actively proliferating and normoxic cells and an inner layer consisting of quiescent and hypoxic (or necrotic) cells.

RNA interference (RNAi) is a sequence-specific, posttranscriptional gene silencing phenomenon that involves the degradation of mRNA.\textsuperscript{15,16} Experimental manipulation of this process is conducted using small interfering RNA (siRNA) molecules that mediate sequence-specific mRNA degradation. RNAi is characterized by high efficiency, sequence-specific gene suppression and simplicity of the experimental procedure. Hence, this technique is widely used as a molecular biology application. The use of siRNA molecules may also have therapeutic potential in cancer through the suppression of genes, which have critical functions in tumor progression.\textsuperscript{17} The systemic delivery of siRNA is currently not possible in a clinical setting, however, some promising strategies to overcome this limitation are under development.\textsuperscript{18}

As described above, HIF-1 and VEGF appear to play an important role in the radiation resistance of tumor cells and are potential therapeutic targets to enhance radiation effects upon certain cancer types. To test this hypothesis more fully, we conducted experiments using multicellular spheroids as our \textit{in vitro} tumor model and RNAi as our method of gene suppression. We established spheroids lacking HIF-1 and VEGF expression as a result of siRNA targeting. We then evaluated the contribution of these genes to the radiation sensitivity phenotype of the spheroids under both normoxic and hypoxic conditions.

\section*{MATERIALS AND METHODS}

\textbf{Cell culture and the establishment of spheroids and mouse tumors}

The SQ5 human lung squamous carcinoma cell line has been maintained in our laboratory for some years. These cells are cultured in alpha-MEM (ICN Biomedical Inc., Aurora, OH) containing 10% fetal bovine serum, 20 mM HEPES, 8 mM NaHCO\textsubscript{3}, 50 U/ml penicillin, and 50 \mu g/ml streptomycin in a humidified incubator at 37°C under an atmosphere of 95% air and 5% CO\textsubscript{2}.\textsuperscript{19,20} For experimental manipulation and analysis, monolayer and spheroid cells were prepared as described below. For generation of a tumor mouse model, aliquots (1.0 \times 10\textsuperscript{7} in 100 \mu l medium) of SQ5 cells were subcutaneously implanted into the backs of 7- to 9-week-old, pathogen-free BALB/c nude mice (Clea Japan, Inc. Tokyo, Japan). Tumor samples were obtained after approximately 15 days, when the lesions were at least 5 mm in diameter.\textsuperscript{21}

\textbf{siRNA transfections}

Commercially available siRNA for HIF-1 and VEGF were obtained (HIF-1A, Silencer Validated siRNA, and VEGF-A, Silencer Pre-designed siRNA respectively, Applied Biosystems, Carlsbad, CA). A control siRNA with no homology to any known mammalian gene was used as a negative control (AAATCTCTCCGACGTGCCTGTCGT, Qiagen, Hileden, Germany). Transfections were preformed as described below and expression vectors were not used.

\textbf{Monolayer cultures.} The forward transfection method was applied for monolayer cells. Briefly, exponentially growing cultures were prepared by plating 8 \times 10\textsuperscript{5} cells in 40-mm culture dishes at 24 h before transfection. Immediately prior to transfection, the culture medium was aspirated and the cells were washed with PBS. This was followed by the addition of 620 \mu l of fresh medium to the cells. RNAiFect Transfection Regent (Qiagen) was used for siRNA transfection according to the manufacturer’s protocol. For each dish, 3.6 \mu l of 20 \mu M (0.25 \mu g/\mu l) siRNA stock solution was diluted in complete media to give a final volume of 100 \mu l. 2.7 \mu l of RNAiFect Transfection Regent was then added to give a ratio of RNAiFect (\mu l): siRNA (\mu g) of 3:1 (20 \mu M stock solution = 0.25 \mu g/\mu l; 3.6 \mu l of siRNA = 0.9 \mu g, i.e. one third of transfection reagent). The siRNA-transfection regent mixture was then incubated for 10–15 min at room temperature to allow formation of transfection complexes and 100 \mu l of the complex was subsequently added to the dish. The final volume of the medium in the wells was 720 \mu l and the final concentration of siRNA was 100 nM. Cells were incubated with the transfection complexes for 24 h.

\textbf{Spheroids.} A reverse transfection method was employed for the introduction of siRNA molecules into spheroids. Twelve transfected spheroids were prepared for each experiment as follows: 6 \mu l of a 20 \mu M siRNA stock solution was diluted in 400 \mu l of Opti-MEM I medium (GIBCO Invitrogen, Carlsbad, CA) without serum. 4 \mu l of lipofectamine RNAiMAX (Invitrogen) and siRNA solution were mixed and incubated for 10 min. Cells (6 \times 10\textsuperscript{5}) were prepared in 2 ml of complete growth medium without antibiotics. The siRNA and lipofectamine complexes were then added to the diluted cells in a 2.4 ml final volume. Aliquots of 200 \mu l of this solution were then added to each of 12 wells of an agarose-coated culture plate (Sumilon, Sumitomo Bakelite,
Tokyo, Japan). Each well thus contained 5 × 10^3 cells and a final siRNA concentration of 50 nM. The cells were incubated for 72 h at 37°C in CO2 incubator until the spheroids reached 500 μm in diameter.

**Hypoxic conditions**

Hypoxic conditions were generated by incubating cells for 6–24 h at 37°C in a Gaspak™ Pouch Anaerobic System (Becton Dickinson and Company, San Jose, CA) or Anaero Pack System (Mitsubishi Gas Chemical, Tokyo, Japan), both of which can rapidly produce hypoxic environments. Culture dishes and plates were shielded in plastic bags containing a deoxygenating agent, and were incubated for 6 h before exposure to radiation.

**Radiation**

Cells were irradiated using a Cs-137 Gamma Cell irradiator (Atomic Energy, Ontario, Canada) at room temperature under both normoxic and hypoxic conditions. Radiation doses were given from 0 to 8 Gy at a dose-rate of approximately 1 Gy/min. After radiation, cells were processed on ice to prevent reoxygenation.

**Colony formation assay**

The cytotoxic effects of siRNA and/or radiation were assessed via a colony formation assay. The size of the surviving cellular fraction was determined as described as previously. Briefly, after treatment with siRNA and/or radiation followed by incubation under hypoxic or normoxic condition as described above, monolayer cells and spheroid were digested in 0.05% trypsin solution containing 0.02% EDTA. Then, cells were counted, and replated in 60-mm culture dishes with 150–1000 cells/dish based upon the dose of radiation and oxygenic condition, at which 100 colonies/dish were obtained. Cells were incubated for 10–14 days to allow colony formation, and then were stained with 0.5% of crystal violet. Clonogenic survival curves were constructed from at least three independent experiments. The plating efficiency for the SQ5 cells was 0.8.

**Extraction of RNA and cDNA synthesis**

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized from these RNA preparations using a SuperScript™ III RT-PCR kit (Invitrogen) according to the manufacturer’s protocol.

**Quantitative real-time PCR**

The levels of HIF-1 and VEGF mRNA in both the monolayer and spheroid cell cultures were measured by quantitative PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Commercially available primer and probe sets from the TaqMan® Gene Expression Assay kit were used (Applied Biosystems). Beta-actin RNA was used as a control to normalize gene expression using the pre-developed TaqMan Assay Reagents (Applied Biosystems). All PCR reactions were performed in 25 μl total volume using TaqMan® Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol.

**Western blotting**

Monolayer, spheroid, and tumor cell lysates were subjected to western blot analysis as described previously. Briefly, for each sample, 25 μg of protein was separated using SDS-polyacrylamide gel electrophoresis. The proteins were then transferred onto a PVDF membrane and incubated with the primary antibody in TBST for 1 h at room temperature (HIF-1α, 1:500; BD Biosciences Pharmingen, San Jose, CA and beta-actin, 1:1000; New England Bio Labs, Beverly, MA). The membranes were then incubated with a secondary antibody (anti-mouse, 1:1000; New England Bio Labs) in TBST also for 1 h at room temperature. The immunocomplexes were visualized using an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and images were developed with LAS-3000 (Fujifilm, Tokyo, Japan).

**Statistical methods**

Results are presented as mean ± standard error (SE) from at least three independent experiments. Comparisons of the surviving fractions were made by Student’s t-test or analysis of variance (ANOVA). All p values < 0.05 were considered statistically significant.

**RESULTS**

**Comparison of HIF-1 protein expression in monolayer and spheroid cultures and mouse tumor xenografts of SQ5 cells**

We first analyzed the intrinsic expression of the HIF-1 protein by western blotting analysis of monolayer and spheroid cultures of SQ5 cells and mice tumor xenografts generated with these cells. As shown in Fig. 1, monolayer cells showed...
barely any expression of HIF-1 protein. In contrast, the spheroid and xenograft cells showed higher expression of HIF-1. This finding suggests that HIF-1 expression is enhanced during the growth of 3-dimensional cell structures.

Effects of siRNA suppression of HIF-1 and VEGF in SQ5 monolayer and spheroid cultures under both normoxic and hypoxic conditions

To further examine the effects of HIF-1 and VEGF on radiation sensitivity in SQ5 monolayer and spheroid cultures, we conducted analyses under normoxic conditions as well as hypoxic conditions in which HIF-1 and VEGF expression would be elevated. We first confirmed whether HIF-1 and VEGF are enhanced under hypoxic conditions, and also if targeted siRNA efficiently suppresses the expression of these genes under both normoxic and hypoxic conditions. As a control, cells were transfected with a non-targeting siRNA instead of HIF-1 or VEGF siRNA. Monolayer cells were treated with 100 nM of siRNA for 24 h and then exposed to hypoxic conditions for 6 or 24 hours. For spheroid cultures,

![Graphs showing effects of HIF-1 and VEGF siRNA on monolayer cells and spheroid under normoxic and hypoxic conditions. (a) SQ5 monolayer cultures. The expression of HIF-1 and VEGF appeared to be enhanced under hypoxic conditions for 24 h (open bars) but completely down-regulated by the corresponding siRNA (closed bars). (b) SQ5 spheroids. HIF-1 and VEGF expression also increased under hypoxic conditions for 6 h (open bars), which was again blocked by the corresponding siRNA (closed bars).]
Suppression of HIF/VEGF and Radiosensitivity

Single cells were treated with 50 nM siRNA using the reverse transfection protocol and grown as spheroids for three days as described in the materials and methods section. These cultures were then incubated for an additional 6 hours under hypoxic conditions. Gene expression was estimated by quantitative RT-PCR. Beta-actin expression was used as a normalization control. As shown in the left panel of Fig. 2a, hypoxic conditions appear to affect the expression of HIF-1 mRNA in monolayer cells; these levels were slightly decreased under hypoxic condition after 6 hours but then obviously enhanced after 24 hours (open bars). This expression was almost completely suppressed by pretreatment with HIF-1 siRNA (closed bars). Comparable results were observed for VEGF in the same experiments (Fig. 2a, right panel) i.e. enhanced VEGF expression was observed under hypoxic conditions but down-regulated by siRNA. However, suppression of VEGF was lesser extent compared with HIF-1.

As shown in Fig. 2b, the expression of HIF-1 or VEGF mRNA in spheroids also increased under hypoxic conditions for 6 hours and this enhanced expression was down-regulated by treatment with the corresponding siRNA.

The effect of siRNA on HIF-1 protein expression was next confirmed by western blotting. Monolayer and spheroid cultures of SQ5 cells were incubated both under normoxic and hypoxic condition for 6 hours. As a control, cells were transfected with a non-targeting siRNA instead of HIF-1 siRNA. As shown in the left panel of Fig. 3, monolayer cells show no expression of HIF-1 under hypoxic condition. In addition, whereas the abundant expression of HIF-1 protein is induced under hypoxic condition, this is almost completely inhibited by pre-treatment with HIF-1 siRNA for 24 hours. In the spheroid cultures, HIF-1 protein expression was detectable under normoxic conditions, and enhanced under hypoxic conditions. HIF-1 protein expression under both conditions were inhibited completely by siRNA treatment (right panel of Fig. 3). These results are therefore consistent with the RT-PCR data described above.

Cytotoxic effects of the combination of HIF-1/VEGF siRNA and radiation on SQ5 monolayer and spheroid cultures under normoxic and hypoxic conditions

We next examined the effects of HIF-1 or VEGF siRNA on the sensitization of SQ5 cells to radiation. Cells were treated with HIF-1 or VEGF siRNA and exposed to hypoxic condition for 6 h. These cells were then treated by 2–8 Gy of radiation. Cytotoxic effects were determined by colony formation assay. Treatment with HIF-1 siRNA slightly enhanced the radiation-induced cell killing (closed triangles). However, the difference in survival between the presence and absence of siRNA at each dose of radiation were not significant. VEGF siRNA had no impact upon the cytotoxic effects of radiation in this experiment (closed circles).
on the radiation cytotoxicity levels in our monolayer and spheroid cultures by colony formation assay. The cells were treated with HIF-1 or VEGF siRNA and exposed to hypoxic conditions for 6 h. The cells were then exposed to 2–8 Gy of radiation. Cytotoxic effects were determined by colony formation assay. a) Normoxic conditions. Neither HIF-1 nor VEGF siRNA enhanced radiation cytotoxicity. b) Hypoxic conditions. The same result was obtained as shown for normoxia.

Monolayer cells

The sensitivity of SQ5 cells to radiation alone was estimated using cells transfected with the control siRNA. Cells incubated under hypoxic conditions appeared to be more resistant to radiation than the cultures grown under normoxic conditions (open squares and open diamonds in Fig. 4, respectively). HIF-1 protein expression in the monolayer cells was not observed under normoxic condition but was found to be induced under hypoxic conditions (Fig. 3). Hence, the effects of siRNA on radiation cytotoxicity were examined under hypoxic conditions only. Treatment with HIF-1 siRNA slightly enhanced radiation-induced cell killing slightly (closed triangles in Fig. 4). However, the difference in survival between the presence and absence of siRNA at each dose of radiation were not significant. VEGF siRNA had no discernible effects on the radiation cytotoxicity (closed circles).

Spheroid

In contrast to the monolayer cultures however, the spheroids showed expression of HIF-1 protein under both normoxic and hypoxic conditions. Hence, the effects of HIF-1 and VEGF siRNA treatment on the radiation sensitivity of spheroids were examined under both sets of conditions (normoxic, Fig. 5a; hypoxic, Fig. 5b). In a similar manner to the monolayer experiments, the sensitivity of the spheroid cultures to radiation alone was first estimated using cells transfected with control-siRNA. As expected, spheroid cells under hypoxic conditions appeared to be more resistant to radiation than those under normoxic condition (open squares in Figs. 5a and 5b). As shown in Fig. 5a, under normoxic conditions, neither HIF-1 nor VEGF siRNA alone affected the radiation cytotoxicity levels in spheroids compared with radiation alone. Similar results were observed under hypoxic conditions (Fig. 5b), indicating that the down-regulation of HIF-1 or VEGF does not influence the cytotoxic effect of radiation in tumor cells under either normoxic or hypoxic conditions.

**DISCUSSION**

Hypoxia-induced radiation resistance is a principal cause of cancer treatment failure after radiation therapy. In this regard, HIF-1 and VEGF may play key roles in regulating the biological responses to hypoxia in tumor cells. We speculated that down-regulation of the functions of these genes may conquer radiation resistance to tumor cells under hypoxic conditions. To elucidate this possibility, we tested whether gene knockdowns using HIF-1 or VEGF siRNA would enhance the cytotoxic effects of radiation on cancer cells under either hypoxic or normoxic conditions. We employed spheroid cultures of SQ5 lung carcinoma cells as our solid tumor model for these analyses. As expected, a hypoxic environment increased the expression of HIF-1 and VEGF and enhanced the surviving fraction of cells in the spheroids after radiation exposure. However, HIF-1 or VEGF gene
knockdown with siRNA did not enhance these cytotoxic effects of radiation in our experiment. Our results thus indicate that neither HIF-1 nor VEGF play a critical role in the tumor cell response to radiation, or that the core functions of these genes in this regard are compensated for upon their down-regulation.

There are several reports that have now investigated the role of HIF-1 in radiation sensitivity in cancer cells in vivo and in vitro. The results of such in vitro experiments including our current analyses are somewhat controversial however. Vordemark et al. have shown that the HIF-1 protein accumulation levels under hypoxia did not always correlate with cellular radiation resistance i.e. a cell type-specific association was observed. Arvoid et al. have reported that the cytotoxic effects of radiation on HIF-1-negative or -positive cells are identical under either hypoxic or normoxic conditions. These authors concluded that hypoxia-mediated radiation resistance is independent of HIF-1 which is consistent with our current findings in spheroids. In addition, they used the 2–8 Gy radiation dose and clonogenic assay in their studies, which was also used in our current study. On the other hand, under similar experimental conditions, Moeller et al. have previously reported different results to those presented herein. These authors have reported that HIF-1 inhibition enhances the cytotoxic effects of radiation (up to 12 Gy) in a clonogenic survival experiment under hypoxic conditions.

Several previous in vivo experiments using animal models have also presented data which indicates that the down-regulation of HIF-1 expression produces an enhancement in the anti-tumor effects of radiation. For example, Zhang et al. have reported that the intratumoral injection of adenovirus encoding HIF-1 siRNA resulted in a significant inhibition of tumors with growth after radiation. Williams et al. have also demonstrated previously that mice xenograft tumors derived from a HIF-1 deficient cell line showed an enhanced response to radiation compared with the corresponding parental tumor cells. Of interest in this regard, no difference in the radiation response of same paired cell lines was observed in vitro status. Several other studies have suggested that the relationship between radiation sensitivity and the biological effects of HIF-1 are complex i.e. not all HIF-1-mediated effects are associated with radiation resistance in tumors. They have suggested that HIF-1 inhibiting strategy would have an overall positive or negative impact on tumor radiosensitivity.

Many anti-VEGF strategies including the use of VEGF receptor tyrosine kinase inhibitors or VEGF antibodies result in an enhancement of radiation effects on tumor cells. The main target of VEGF is tumor-associated endothelial cells. Hence, in previous analyses of this gene, tumor mouse models were employed in which endothelial cells and a vascular system is present. On the other hand, and interestingly, some in vitro studies have demonstrated that VEGF receptor tyrosine kinase inhibitor shows direct effects upon the radiation sensitivity of tumor cells themselves, and that this is not exerted through anti-vascularization effects. It is suggested that such agents directly inhibit cell survival signaling pathways in tumor cells, resulting in an increased apoptotic response after radiation.

According to previous studies, the contribution of HIF-1 and VEGF to radiation sensitivity in cancer cells may differ between in vitro systems and animal models. The lack of a radiation enhancement effect in spheroids following HIF-1 and VEGF siRNA in our current analyses, which is in contrast to the results of many published studies, might therefore be due to the chosen experimental model and radiation treatment schedule. Our experimental conditions do have some noteworthy limitations. First of all, we used a spheroid culture system that mimics the three-dimensional structure of solid tumors but lacks the microenvironment that would exist around tumors in vivo including a vascular system. We also applied only single doses of radiation and not a fractionated schedule by which it is possible to assess reoxygenation and repopulation in which HIF-1 and VEGF may play important roles. Further studies using tumor animal models and different radiation schedule are therefore warranted.

In addition to genetic manipulation of HIF-1, several studies have shown that certain chemical agents that interfere with the function of HIF-1 itself and its downstream pathways also enhance the toxic effects of radiation. Significantly, many existing anticancer drugs that were not developed as HIF-1 inhibitors have now been reported to inhibit this gene. Of note also, even though these chemical agents are not sequence specific inhibitors like siRNA molecules, they have advantages in clinical applications because drug delivery is far easier to undertake than gene therapy.

Very few reports have described the siRNA transfection into spheroid systems. Spheroids are characterized by a high cell-density and a closed packed three-dimensional tumor like structure, which leads to severe diffusion limitations for molecules. We could not therefore deliver siRNA molecules directly into the spheroids using the forward transfection methodology that was used for monolayer cells. We instead applied a reverse-transfection technique in which the spheroids were formed from single cells that had been transfected with the siRNA in advance. Oishi et al. have succeeded previously in delivering stable and functional siRNAs directly into spheroids. To achieve this, a PEGylated polyplex system was used in which siRNA molecules can penetrate multiple cell layers of the solid tumor. This system may have potential clinical applications in the future.

We found from our current experiments that HIF-1 expression in spheroids, similar to mice tumor xenografts, was obviously enhanced compared with the monolayer cultures. Takagi et al. have shown in their earlier study that VEGF is also enhanced in spheroids and in solid tumor
mouse models compared with monolayer cultures. The mechanism underlying this may be an increased number of hypoxic cells. Several studies have shown that the hypoxic cellular fraction increases in the inner part of the spheroid as it grows. In line with this, others and we have now shown that spheroids exhibit radiation resistance compared with monolayer cells (Figs 4 and 5). It is highly possible therefore that the hypoxic environment in inner part of spheroid induces HIF-1 and VEGF expression. It is notable in this regard that spheroids contain a physiological hypoxic fraction even under normoxic conditions. Such a chronic hypoxic fraction is generated gradually and mimics the hypoxic fraction in solid tumors in vivo. This differs from the artificially acute hypoxic environment produced using a gas pack pouch or N2 replacement. Taken together, we contend from our current data and previous studies that spheroids may serve as a good experimental model for solid tumors in future investigations of the roles of HIF-1 and VEGF and/or the behavior of hypoxic fractions in tumors.

In conclusion, we have established a method for transfecting siRNA molecules into spheroid cells and thereby analyzed the influence of inhibiting the expression of HIF-1 or VEGF by siRNA on the radiation sensitivity of these structures. We conclude from our current results also that HIF-1 and VEGF do not play a critical role in radiation sensitivity in spheroids under either normoxic or hypoxic conditions. To more fully elucidate the contribution of HIF-1 and VEGR to radiation sensitivity in tumor cells, further investigations will be required involving an in vivo animal model which provides a tumor vascular system and also a fractionated radiation schedule in which reoxygenation of tumor cells can be evaluated.

ACKNOWLEDGMENTS

This research was supported by 1) The Ministry of Education, Culture, Sports, Science and Technology-Japan, Grant-in-Aid for Scientific Research (C) 17591285, 2) a grant under the 2005 Strategic Research Project of Yokohama City University and 3) The 21st Century Center Of Excellence Program 2005 of Yokohama City University.

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