Development of a stable growth factor suitable for radioprotection

— Drug development-aimed R&D at a basic research institute —

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We have developed a stable growth factor protein that is a promising candidate for a radioprotective drug suitable for treating biological damage caused by high-dose radiation. This stable growth factor, designated FGFC (fibroblast growth factor chimeric protein), demonstrates several advantages over existing drugs. Once approved, it can be stockpiled for radioprotection. We aim to develop this protein into a drug at the highest possible level achievable at a basic research institute.

Keywords : Radioprotection, radiation-induced damage, fibroblast growth factor, FGF, stable, crypt, survival

1 Introduction: Positioning of this paper in *Synthesiology*

This research places as its outcome the creation of a pharmaceutical product from a new protein in the advanced basic research phase, and intends to overcome the phase of R&D known as the "valley of death." To achieve the pharmaceutical product outcome, it is necessary to engage in quality-controlled manufacturing, conduct clinical trials, and receive pharmaceutical approval. This entails the time span of over 10 years and billions of yens of R&D funds. Therefore, it is difficult to overcome the valley of death by a basic research institute alone, and product realization has not been achieved for this pharmaceutical product at the point of writing this paper. Some people think that the product realization of pharmaceuticals should not be a development goal of a basic research institute. However, the author believes that a basic research institute can contribute to product realization by optimizing the direction and stages of the R&D. The importance of protein pharmaceuticals is expanding rapidly, and six of the top 10 products were protein pharmaceuticals in terms of global pharmaceutical sales in 2012. This means that the future basic research for drug discovery cannot be discussed without taking the protein drug discovery process into consideration. Therefore, I think it is important to describe the research and the scenario for protein drug discovery conducted at a basic research institute in *Synthesiology*. This paper will discuss the development phases of the signaling molecule protein FGFC as a radioprotective drug candidate conducted by the Author *et al*.

2 Protection against biological damages by exposure to radiation

When an organism is exposed to radiation, various effects occur, though there may be differences in quality or degree. These are the cleavage of nucleic acids and the denaturalization of biological substances by active oxygen and free radicals that are produced by the excitation of water caused by the energy absorbed by the body, depending on the types of radiation such as alpha ray, beta ray, gamma ray, X-ray, or neutron ray. Many such effects are not favorable to biological activity. Since the organisms evolved to adapt to the radiation in the natural environment including cosmic rays, natural radiation from earth, and radiation derived from substances ingested as food, organisms inherently possess a molecular mechanism to overcome such effects. Therefore, most of the effects of radiations at the level present in the natural environment do not cause problems on the individual level. However, when there is exposure to extremely high level of radiation, damage always occurs in a short period (this is called the acute radiation syndrome, or deterministic effect), and this may override the natural healing ability of the organism, and may lead to death of an individual at the worst. Even at low-level exposure, damages may manifest at certain probability after some passage of time (this is called the late radiation injury, or stochastic effect) (Fig. 1).

Therefore, the primary measures against high-level radiation that occurs resulting from accidents or medical treatments are to block the organism from the radiation by physical means such as keeping a distance from the radiation source, or wearing masks to prevent taking in

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the radioactive substances or radioactive particles into the body. In preparation for situations where physical blockage is not possible, preventative methods using mainly chemical substances have been developed as secondary measures to reduce the effect of radiation on organism. Examples are methods such as using some compound to protect the readily affected biological substances such as nucleic acids by detoxifying the free radicals produced by radiation, or to chelate the radioactive substance that entered the body and to promote excretion from the body. However, these are passive measures.

Recently, third measures that can be called active measures have been developed. These are radioprotective methods using the biological mechanisms where molecules are used to act directly on the cells, which are the building blocks of organisms. In one case, it was found that a group of signaling molecules called the cytokine or cell growth factors, which possess the ability to maintain survival or promote reproduction of cells, show activities that prevent or reduce the radiation effect on cells. If a biological radioprotection method using such a signaling molecule group is combined with electromagnetic isolation, physical isolation, or protection by chemical substances, maximum protection against radiation damage can be expected in total. Therefore, we believe that there is a large potential for R&D in developing the signaling molecule with high protective effect, by mobilizing the latest knowledge of biomedicine as well as findings on signaling molecules (Fig. 1).

We hold a research paradigm where various applications are sought by focusing on the multiple functionalities of signaling molecules and through the clarification of new physiological functions and molecular mechanisms. In this paper, from the perspective of research for signaling molecules to achieve the application to protective drugs, we shall summarize the research so far and discuss future development. In the course of this research, we learned that the scenario for sending the product out to society differed between general drugs and radioprotective drugs. This means that the scenario in which

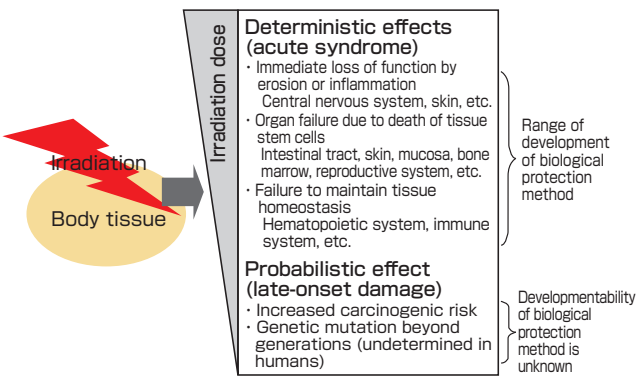


Fig. 1 Biological damage by radiation exposure and room for development of biological protection method

the efficacy is investigated through clinical trials conducted on patient population with target diseases as in general drug development cannot be used in the development of protective drugs for high-dose whole body radiation damage. The development of radioprotective drugs runs into more difficulty than the one for general drugs. In this paper, we describe the R&D scenario at a basic research institute for the drug discovery of radioprotective drugs.

3 Scenario to achieve the outcome of practical protective drugs and the synthesis method for its realization

We engage in the development of a radioprotective drug based on fibroblast growth factor chimeric protein (FGFC), a signaling molecule protein. The details of FGFC will be discussed later (chapter 7).

As a scenario to achieve a protective drug from FGFC, a candidate molecule for a radioprotective drug, we initially considered a linear development. The linear development is the course followed in the development of regular drugs, and it involves the following processes: 1) safety tests of pharmaceutical candidate substances, 2) efficacy trials of pharmaceutical candidate substances in treatment of patients with target diseases (in this research, biological damage by high-dose radiation exposure), 3) application for approval, 4) additional tests and reapplication as needed, and 5) pharmaceutical approval. However, we discussed the feasibility of this development according to this scenario with the physicians and researchers of radioprotection in Japan and overseas, people of the pharmaceutical authorities, and people of World Health Organization (WHO), and reached an understanding that such development was difficult. The main reason was because, there was normally no patient population with radiation exposure that would provide statistically significant analysis, and even if such population existed, it would not be ethically acceptable to set a placebo patient group as a control.

Therefore, we reviewed the scenario to develop the FGFC as a protective drug. Currently, most of the radioprotective drugs used in medical practice at times of emergency exposure accidents have also been shown to be effective as systemic radioprotective drugs that were developed as drugs for some other disease. Prior examples include the keratinocyte growth factor (KGF, will be explained later) and granulocyte macrophage colony-stimulating factor (GM-CSF) that were approved in the United States for the treatment of side effects of cancer therapy. In this research, such prior examples were positioned as scheduled composition, and we restructured the scenario for protective drug development in two stages (Fig. 2). In the first stage, the pharmaceutical approval will be obtained as a treatment drug for a patient group that actually exists, and in the following second stage, the use

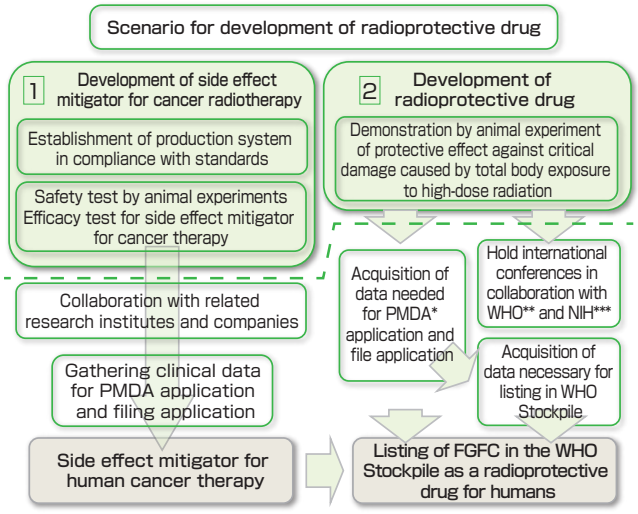
as a radioprotective drug will be achieved. Following the prior examples, the first stage for FGFC was positioned as the development of an agent mitigating side effects that would be approved as a drug to reduce the side effects of cancer therapy. In the following second stage, this drug once approved will be developed as a systemic radioprotective drug (Fig. 2).

3.1 Development of a drug to mitigate the side effect of cancer therapy using radiation

3.1.1 Course of development

The structure of this development policy follows the course of development of FGF7 (also called KGF), approved in the United States, that is used clinically as a side effect mitigator of cancer therapy and pharmaceutically.

The patient who receives chemoradiation therapy for cancer is exposed to a high dose of radiation. Of course, irradiation measures are taken to minimize the damage to normal tissues, but significant degrees of side effects do occur. Particularly, in cases where irradiation is done for head and neck cancer, severe erosion of the oral cavity mucosa occurs, and the patient complains of strong pain and becomes incapable of ingesting food or water. This is the major problem in this therapy. The FGF7 is administered preventatively or *post facto* to patients receiving such treatment, and has shown to greatly reduce the side effects and raise the patients' quality of life (QOL), and as a result increase the effect of cancer therapy. For drugs to mitigate side effects of the cancer therapy, because there exist patient groups in which the pharmaceutical efficacy can be investigated, clinical trials can be conducted. Therefore, we plan to analyze the efficacy of FGFC by evaluating the mitigation activity against side effects in normal tissues of cancer patients receiving the radiotherapy.



*PMDA: Pharmaceuticals and Medical Devices Agency: The institution in charge of the screening and approving drugs and medical devices based on the Pharmaceutical Laws of Japan.
**WHO : World Health Organization: Organization established as a specialized institute based on the UN Charter to promote and protect people's health.
***NIH : National Institute of Health: The institution for biomedical research in the United States. It consists of multiple specialized research centers and supporting organizations. It also allots the research funds.

Fig. 2 Scenario to develop FGFC as a practical radioprotective drug
Consists of Phase 1 and Phase 2. The pale green area above the broken line is the part that can be conducted by a basic research institute.

3.1.2 Establishment of the production system toward approval

Whether it is a side effect mitigator for cancer therapy or a radioprotective drug for high-dose exposure of the whole body, the important common issues are the production and approval of the substance that possesses the quality required as a pharmaceutical. It is necessary to establish a system for mass-producing FGFC at high quality and stability using a method in compliance with the good manufacturing practice (GMP) standards. Then it is necessary to conduct the tests for safety and efficacy for the proteins produced by the established production system, and obtain approval as a drug.

3.1.3 Phase of pharmaceutical approval – Safety and efficacy tests and approval

The drugs used for humans must be safe. Therefore in the development of drugs, the presence of major problems to health is first checked through animal experiments. In the safety test conducted with animals for this purpose, it is required that the same drug administration route used for humans is employed. The protein formulation such as FGFC readily decomposes or is deactivated by digestive enzymes, and since the absorption in the digestive tract and efficiency of transition to blood are extremely low, oral administration is not suitable. Therefore, the protein formulation that must be activated systemically is generally administered intravenously, subcutaneously, or intramuscularly in humans.

After the safety in animals is confirmed, next, the safety in humans is investigated by first phase clinical trial conducted to healthy adult volunteers. If the safety is confirmed, the efficacy as a side effect mitigator of cancer therapy will be demonstrated in a clinical trial. If results suitable as a drug is obtained in the safety and efficacy tests, application is filed with the pharmaceutical authorities. When the approval is obtained, the side effect mitigating drug for radiation therapy is realized. However, large amounts of time and money are necessary to conduct trials in humans, and this surpasses the scale that can be undertaken by a basic research institute alone. Therefore, it is mandatory to form an alliance with external organizations such as pharmaceutical companies or NPOs. The optimization of the production method in compliance with the standards and safety and efficacy tests are done as a joint development with such external organizations.

3.2 Development of radioprotective drug for high-dose exposure to the whole body

3.2.1 Difficulty of conducting the efficacy test in humans

It is mandatory to demonstrate the efficacy as a radioprotective drug for high-dose exposure to the whole body. However, normally, a population of patients who have received whole body exposure of high-dose radiation do not normally exist, and it is difficult to investigate the efficacy in humans using the method generally used in drug development, such as comparing the improvement of symptoms between the two groups that were administered either the candidate drug or placebo. Therefore, it is important to demonstrate the protective efficacy against serious damage by whole body exposure to high-dose radiation mainly by animal experiments.

The investigation of efficacy in humans and the course to drug approval differ greatly from general drugs, as mentioned earlier.

3.2.2 Listing in the stockpile item recommended by WHO

The radioprotective drugs are not for treating general diseases, but are used in special situations. Moreover, the patient population in which the efficacy can be confirmed and the occurrences are extremely limited. It is difficult to grasp the market size. It is therefore thought to be difficult to objectively present the efficacy, which is the precondition to develop the product as a drug for a private company or research institution alone, or to present calculations that show the economic feasibility as a product. With this background, for the effective radioprotective drug for use in radiation accidents, WHO selects and designates effective items in a list named the "Stockpile List for Radiation Emergency" (hereinafter, will be called the WHO Stockpile) and reviews it once every few years. The last WHO Stockpile was created in 2007, and the stocking of the drugs according to this list is recommended for radiation organizations around the world. The WHO recommends that the facilities stock the items in the list in the "amount sufficient to treat 200 people for 10 to 12 days." The necessary stockpile around the world as calculated from this figure is fairly large. Also, since biopharmaceuticals of protein formulation have a relatively short effective period, a regular update of the stockpiled item is necessary. Therefore, many people think that the manufacture and sales of the radioprotective drug stockpile will be feasible for private companies and will contribute sufficiently to industry. The author thinks so, too.

Since 2007, the environment surrounding the radioprotective drugs is changing due to scientific advances and appearance of newly approved drugs. We learned that the WHO is thinking that it is time to review the stockpile list. Therefore, we set as a goal to have the FGFC placed in the WHO Stockpile as a radioprotective drug for humans. To achieve this goal, the important future issue is to appeal the efficacy of FGFC to the radiation specialist communities at places such as international conferences.

4 Research objective and outcome: Scenario and strategy for the development of radioprotective drug – Use of a signaling molecule

Research objective: To develop a radioprotective drug using a signaling molecule in order to prevent as much as possible the biological damage caused by high-dose radiation exposure, to treat the damage that has been caused, and to restore a healthy body. Also, to provide the protocol for using this drug.

4.1 Scenario for radioprotective drug development particularly for internal exposure

Assuming a situation that requires a radioprotective drug after a radioactive substance has been taken into the body (internal exposure), the scenario for protective drug development can be set relatively easily. That is, the following measures are necessary:

a. To expel the radioactive substance that entered the body, and
b. To prevent the radioactive substance that entered the body to become incorporated into the target organs and cause damage.

Currently, among the protective drugs designated as the stockpile items of radioprotective drugs, the measures for "a" include Prussian blue and diethylene triamine pentaacetic acid (DTPA), and the measure for "b" include potassium iodide.

Since the Prussian blue and DTPA of "a" have the characteristic of bonding with the radioactive cesium or plutonium, when the person who ingested such radioactive substances is orally administered such protective drug, a cohesion is produced in the digestive tract and the substance is excreted from the body. The damage to the cell is reduced by reducing the time such radioactive substances remain in the body. On the other hand, potassium iodide of "b" utilizes the fact that the chemical form is the same as the radioactive iodine in the body of the person who ingested it.

Since radioactive iodine is highly volatile, it disperses in the atmosphere as gas and enters the blood through respiration. It is then likely to be incorporated into the thyroid gland, which is the organ that produces hormone using iodine as the important component. It is thought that the occurrence of thyroid cancer in children may increase due to this effect. Therefore, if the exposed individual takes non-radioactive potassium iodide, the incorporation of radioactive iodine to the thyroid gland can be reduced greatly.

As it can be seen, even though there is a difference in individual and tissue level between “a” and “b,” both are protective drugs where the principle is to reduce the damage to the body by reducing the exposure through distancing the radioactive substance from the body.

4.2 Scenario for signaling molecule development as a biological protection drug independent of the exposure form

Regardless of whether the exposure is external or internal, it is necessary to conduct effective protective measures in case high-dose exposure cannot be avoided. The damage by exposure to radioactive substances that are incorporated into the body is the same as the damage by external radiation, excluding the point that the distance between the radiation source and the target tissue is short. The following active mechanisms of radioprotective drugs are thought to counter the damage:

a. To prevent the denaturalization of cell components by radiation, such as DNA damage or cell death,
b. To restore the cell components that was denatured, such as DNA that was damaged by radiation, and to prevent cell death, and
c. To promote growth and differentiation of the surviving healthy cells to supplement the cells that died due to radiation.

Of these, free radical scavenger “edaravone” can be given as an example of protective drugs that is a chemical substance with mechanism of “a.” Similarly, it is thought that substance that enhance production and activity of superoxide dismutase (SOD) that is the antioxidant enzyme in the cell acts to counteract the free radicals. It is also thought that the denaturalization of biological molecules by radiation occurs indirectly through the production of free radicals by radiation, and the scavengers that counteract such activity are widely effective.

On the other hand, mechanisms “b” and “c” are mainly the function of biological radioprotective drugs. The biological radioprotective drugs that are currently used in practice include the signaling molecules (bioactive proteins that are created by the cell of the body and acts on the cells) that act on the blood cells and immune systems. The granulocyte colony stimulating factor (G-CSF) is an example. The G-CSF is a signaling molecule that acts only on the growth differentiation of the blood cells, or the free cells that function in the blood or lymph such as erythrocyte, leucocyte, macrophage, and others. This acts to improve the aplastic blood cells. Also, other development candidates include the signaling molecules that target the blood and immunity cells such as thrombopoietin (TPO) receptor agonist, erythropoietin (EPO), interleukin (IL)-3, IL-7, and IL-11.

However, somatic cells such as the intestinal mucosal cell, vascular endothelial cell, hepatic cell, and fibroblast, which are the main cells that constitute the organs that are affected readily by high-dose radiation and may acutely threaten the life of an individual, have origins and functions that differ greatly from the blood cells, and the aforementioned signaling molecules do not function. The inability to use the molecules to protect such cells against radiation damage is a major problem, and the development of signaling molecules with such activities must be done quickly. Of course, the scenario may involve the maximization of radioprotective effects by combining the two.

5 Deepening of the scenario: Selection of the signaling molecule FGF – Selection of FGF1 and the issue of overcoming instability

In the aforementioned situation, it was reported that “Palifermin,” a drug that was approved by the US pharmaceutical authorities for the treatment of oral mucosal inflammation resulting as a side effect of chemoradiation therapy for cancer, was effective as a radioprotective drug.

In fact, this drug was part of the family of fibroblast growth factors (FGF), for which we have been conducting basic research over the years. It is a molecule called the FGF7 (KGF). The FGF family consists of 22 types of genes/proteins, from FGF1 to FGF23, and the molecule have similarities and differences in structure and bioactivity. We thought that the FGF family would have high potential as a radioprotective drug. Therefore, we proposed a research plan with the objective of developing a highly effective radioprotective drug to prevent and treat biological radiation damage using the FGF activity, and this plan was selected by the Budget for Nuclear Research of the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

First, the factors expected to have radioprotective activity among the 22 molecules existing naturally as the FGF family were investigated, and their activities were compared in animal experiments using mice. The cell damage of intestinal crypt, which may be critically damage enough to threaten life, was selected as the analysis item of radiation damage, and the radioprotective activity was compared using this item as an index. It was found that compared to FGF7 and FGF10 that had similar activity to FGF7, FGF1 showed stronger radioprotective effects (Fig. 3).[1]

However, FGF1 had a disadvantage in using it as a pharmaceutical drug, because the natural form of FGF1 was unstable physicochemically and bioactively. Therefore, the development of stabilized FGF (FGF1) arose as a technological challenge that had to be overcome.

6 Scenario for the development of stabilized FGF: Part 1 – Development of PG-FGF1 and the issues due to its novelty

We aimed for the pharmaceutical application of FGF1 from various bioactive aspects, but reached the understanding that the greatest issue in its application was its low stability. Therefore, we attempted the stabilization of FGF1 through various approaches. The molecule group PG-FGF1 that was planned and created based on the scientific findings was our prime result.

To explain the molecular structure of PG-FGF1, we must first explain the mechanism of the FGF action. The FGF binds to the extracellular domain of the FGF receptor that is exposed on the surface of the target cell. This causes the structural change of the receptor, and activates an enzyme called tyrosine kinase on the extracellular domain of the FGF receptor. In that reaction, it is necessary to acquire the cooperation of the sugar chains on the cell surface to obtain the optimal activation and strong binding with the FGF molecule receptor (Fig. 4).

This sugar chain belongs to the category called the sulfated glycosaminoglycans, and belongs mainly to the molecular group called heparan sulfate. Here, it is called a molecular group because it shares similar sugar chain skeletons with diverse microscopic structures such as sulfates. The biological protein covalently bound to sugar chains, such as heparan sulfates, is called proteoglycan (PG). One of the biological importance of this sugar chain is that the structure and activity of FGF can be stabilized through the binding of heparin sulfate sugar chain and FGF. Therefore, to stabilize the structure and activity of the FGF1 protein, we considered binding the protein and heparin sulfate through covalent binding without depending on the force of the molecules on the cell surface. Therefore, we succeeded for the first time in the world to create a single molecule of proteoglycan and FGF1, and named this PG-FGF1. The PG-FGF1 has been shown to have ideal property as a drug, such as an increased activity in the inflammatory environment as well as a stable property (Fig. 5).[2]-[10] It is thought that the property of this molecule is ideal also as a radioprotective drug.

However, there were issues to be solved in using the PG-FGF1 as a radioprotective drug. Roughly divided, the issues are the problem of pharmaceutical approval including quality control, and the technological issue for its production. These issues are universal technological issues accompanying the production of complex carbohydrates (or the majority of the glycan pharmaceuticals), and a long time is needed for solving the basic issues. Therefore, as Scenario 2 in this study, we decided to select the FGFC with less unsolved issues in production processes. The details of this process will be described in some other occasion and will not be addressed in this paper.

We changed the development scenario drastically, and decided to develop a radioprotective drug based on the highly stable FGF (FGFC will be described below) for which the development as a protective drug is expected to be accomplished in a short time because it is a simple protein that can be produced by *E. coli*. The following chapters will describe FGFC. We also think that both PG-FGF1 and FGFC will be better than the current FGF drug when used as radioprotective drugs. If both drugs are created, we expect the PG-FGF1 to have higher efficacy due to the superior principle. Following this way of thinking, if a radioprotective drug using the current FGF drug is considered to be the first generation, FGFC can be set as the second generation, and PG-FGF1 the third generation (Fig. 6).

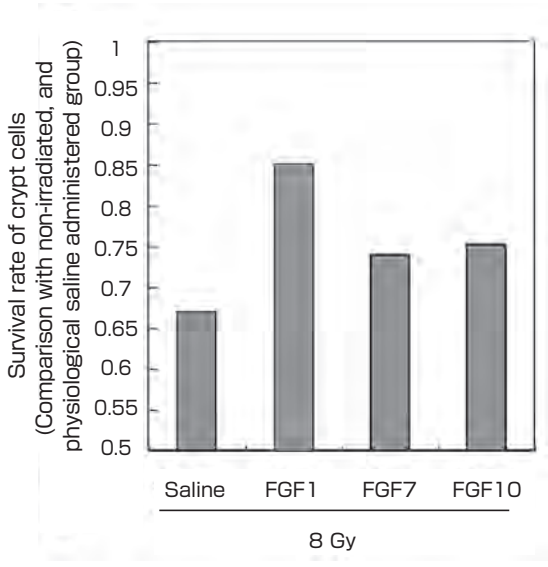


Fig. 3 Comparison of survival rate of intestinal crypt cells 2.5 days after exposure when various FGFs are administered before radiation exposure

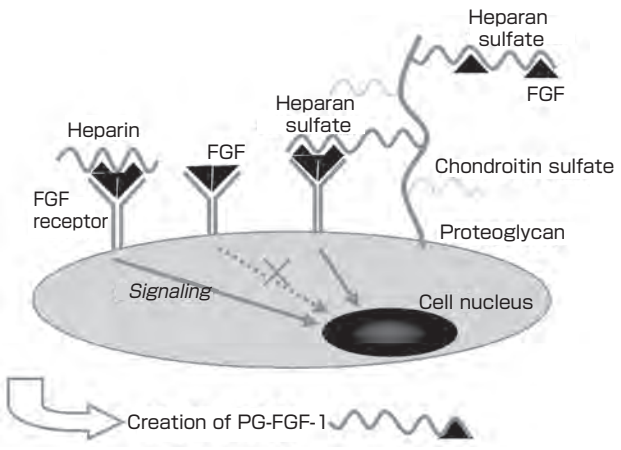


Fig. 4 For the activation of growth factor FGF signaling, the coexistence of glycosaminoglycan sugar chains such as heparan sulfate is mandatory. PG-FGF1 is a molecule in which FGF1 and heparan sulfate are united.

7 Scenario for the development of stabilized FGF: Part 2 – Development of FGFC and its property

7.1 Idea for FGFC

As mentioned in chapter 5, while the PG-FGF1 was fabricated in a logical approach with the objective of creating a superior-function FGF based on scientific findings, FGFC was a high-function molecule obtained by luck. FGFC is an artificial protein produced using *E. coli* and chimerization of a number of FGFs in a cassette format. Since there are multiple combinations for chimerization, here, we use the term FGFC as the general name for several molecular groups.

The basic idea for FGFC was born back in 1988. At that time, the evaluation for the use of FGF as a pharmaceutical was undetermined, and its use as a radioprotective drug was not considered at all. I started the research of molecular biology as a visiting researcher at an American laboratory where Dr.

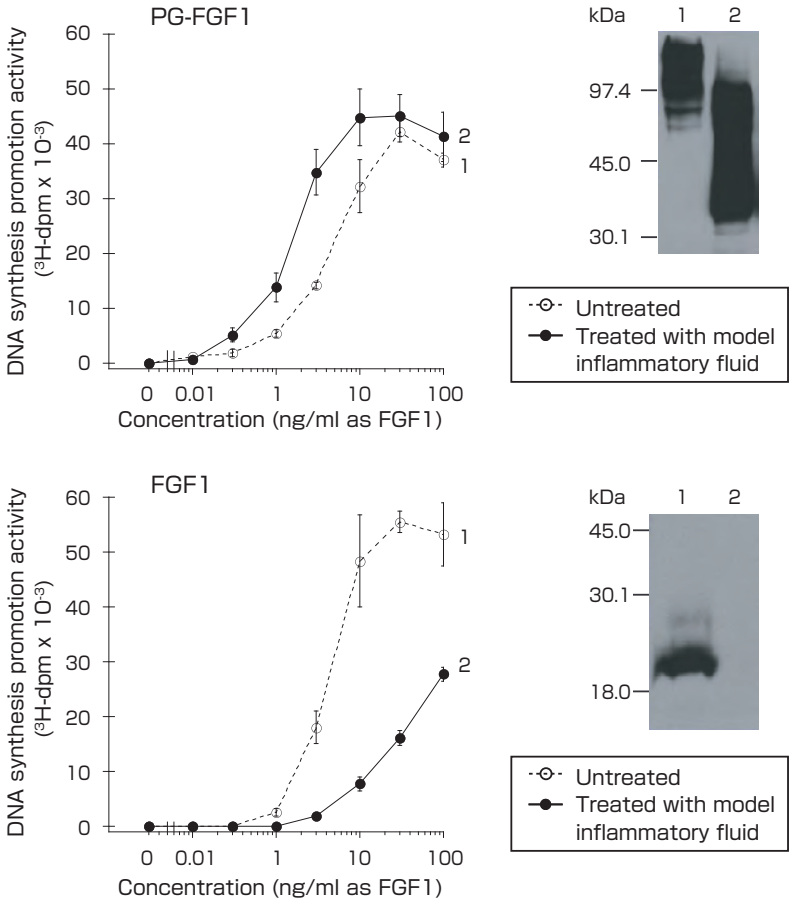


Fig. 5 The activity of PG-FGF-1 is strengthened in the inflammatory environment (top left). This is thought to occur as the sugar chain decomposes partially and functions as an activator of the FGF1 (top right). The natural form FGF1 is decomposed by the enzyme in the inflammatory liquid (lower right), and loses its activity (lower left).
[Data taken partially from Yoneda *et al.*, *Nature Biotechnology* (2000)]

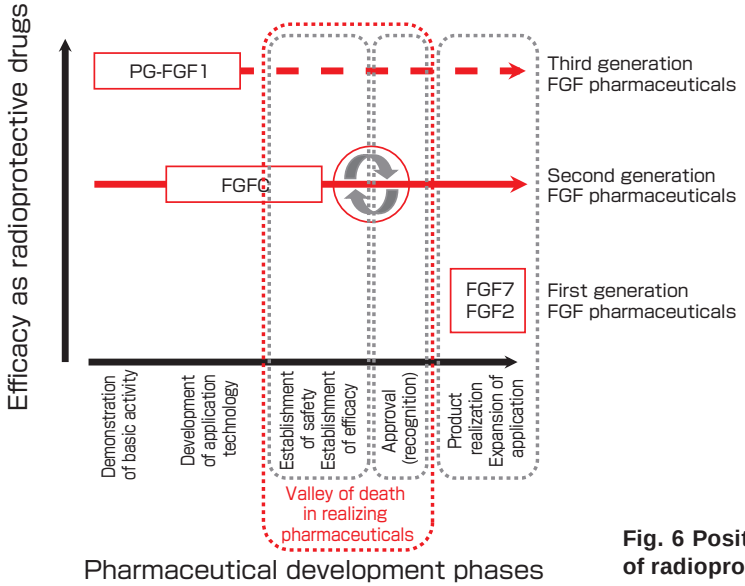


Fig. 6 Positioning of PG-FGF1 and FGFC in the development of radioprotective drugs that employ the FGF activity

Maciag discovered FGF1. At the time, the primary structures of FGF1 and FGF2 had just been clarified. In the research I had done in Japan, I had found that there were similarities and differences in the properties of FGF1 and FGF2, and in the US, I worked on the research to clarify the molecular structures of FGF1 and FGF2 that would be the foundation of their properties. I constructed several types of artificial genes (cDNA) of FGFC by synthetic oligonucleotides and a cassette shuffling method, translated them into protein, and conducted bioactivity analysis of a number of the resulting proteins. During the research, I encountered many problems such as the limitation of experimental methods available at the time and low reliability of the nucleic acid synthesizer, but I was able to complete the construction of the genes for all FGFC as initially planned.

7.2 Establishment of the mass production system for FGFC

The reason FGFC is superior to PG-FGF1 at this point in terms of practical use is that it is a simple protein that can be easily mass-produced using *E. coli* or other prokaryotic expression systems. I shall not go into details, but currently, the production of FGFC protein in labs is done using the *E. coli* equipped with T7 bacteriophage and a plasmid vector called pET-3c. This protein expression system is a type called "*E. coli* hijacking system," and while it is widely used around the world, I was lucky that I received and was able to actually use the materials and information early from the researcher who developed this system. Using this system, it became possible from the early stages to prepare the recombinant protein in the lab, at the scale of several ten to several hundred times more than the conventional recombinant protein expression method. Therefore, it was possible to produce large amounts of various FGFCs and to conduct various analyses for their activities and properties.[11][12]

7.3 Rediscovery of FGFC efficacy from the receptor bond specificity

By analyzing the responsivity of various FGFCs using various cultured cell types, we were able to select the few types that showed characteristic properties and bioactivity. For some molecules, we found that there was high activity

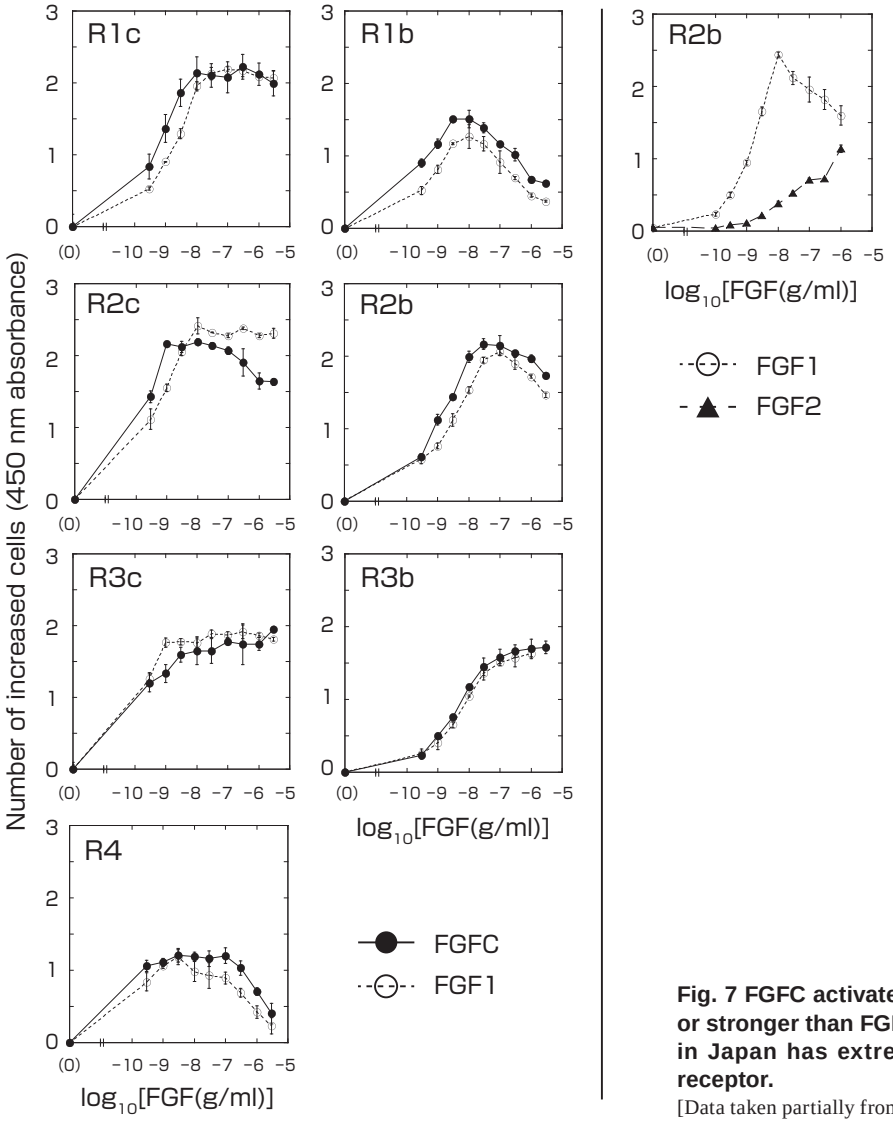


Fig. 7 FGFC activates all types of FGF receptors equally or stronger than FGF1. The FGF2 that is a drug approved in Japan has extremely low activity against the R2b receptor.
[Data taken partially from Motomura *et al*., *BBA* (2008)]

without dependence on heparin. These molecular structures were the basic form of the stabilized FGF with a specific structure that we call FGFC today.

When a cell detects FGF on its surface, there is an involvement of a transmembrane protein called the tyrosine kinase receptor, and the extracellular part of the receptor specifically detects and binds with the FGF molecule. Then the enzyme tyrosine kinase resides in the intracellular component of the receptor becomes activated. The tyrosine kinase FGF receptor transmits the presence of extracellular FGF as an intracellular signal. To describe the FGF activity precisely in the molecular level, it is necessary to manipulate the receptors experimentally. There are four types of tyrosine kinase FGF receptor genes, and these genes code the total seven main types of FGF receptor proteins. Therefore, a cell based screening system was created to analyze the signaling by each receptor. As a result of analyzing the receptor specificity using this experimental system, we were surprised to find that the basic FGFC possesses the ability to activate all seven types of FGF receptor proteins to the same degree or slightly stronger than FGF1. This is a property unseen in other natural form FGFs (Fig. 7).[13]

On the other hand, the bioactivity of a basic FGFC was the same as FGF2 in the property that it was not influenced greatly by the coexistence of heparin sugar chain. Next, we investigated the stability of the three-dimensional structure of the protein needed to express the FGF activity, using the melting point. It was found that in the condition of the investigation, the melting point of the basic FGFC was about five degrees higher than FGF1. From these results, it was strongly suggested that the basic FGFC has higher stability than FGF1 and has a superior property as a pharmaceutical compound.

7.4 Optimization of the FGFC structure with an eye on pharmaceutical use

It was found that the basic FGFC had a wide range of specific bioactivity, was also stable, and this molecule was an excellent candidate for pharmaceutical use. We attempted further optimization of the molecular form. That is, the optimization of the fine structures were done for the purpose of two objectives: minimizing the antigenicity against humans that could not be pursued earlier for the FGFC molecular group created earlier due to the technological limitations at the time, and optimizing the resistance to protein dissolving enzymes. In the initial molecular form, the recognition sequence of the limiting enzyme was introduced to the gene to maintain the seam for chimerization, and there was a concern for the antigenicity against humans as partial amino acid replacement could not be avoided. However, in the current molecular engineering, the amino acid sequence can be designed freely, and therefore we fabricated several types of molecules using the primary structure of the prototype FGFC as a base, and, for example, eliminated the amino acid sequence other than those of FGF1 or FGF2. In this maneuver, we selected a molecule with the highest resistance to the degradation by proteolytic enzymes. This is the current FGFC. Thus, FGFC of optimized molecular structure with an eye on pharmaceutical use was established (Fig. 8).[13]-[15] In this paper, this molecule will be called FGFC.

In the sequence of this FGFC, there is no amino acid introduced artificially to chimerize the two types of proteins that originally exist in humans. Therefore, the antigenicity when it is administered to humans is expected to be minimal, but the actual antigenicity test has not been done. This test will be conducted as one of the items of the safety tests.

8 Large potential of FGFC as a radioprotective drug candidate

8.1 Protection of intestinal damage (prevention through preliminary administration)

One of the major causes of life threatening damage by high-dose radiation is the loss of intestinal function due to the death of the stem cell clusters (crypts) in the intestinal mucosal cells. This is because the intestinal tracts maintain its structure and function by supporting the cell metabolism through incessant regeneration of the cells. As mentioned

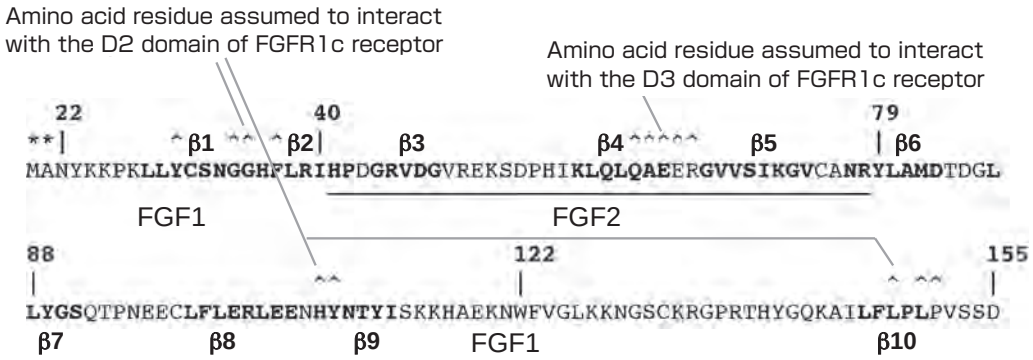


Fig. 8 Primary structure of FGFC
The structure is composed of the sequences derived from FGF1 and from FGF2.

earlier, FGF1 had the highest radioprotective effect among the natural form FGFs. Therefore, the protective action of FGF1 and FGFC were compared. The experimental mice were peritoneally administered either of the FGFs, 10 Gy of gamma ray was irradiated 24 hours later, and the number of live cells in the crypt was counted 3.5 days after. As a result, the FGFC administered group showed significantly higher number of cells compared to the FGF1 administered group. Of course, it was much higher than the control group that was not administered any drug. Therefore, it was shown that the FGFC was superior to FGF1 in the protective action against intestinal radiation damage (Fig. 9 left).[15]

That was not all. Since the bioactivity of FGF1 necessitated the presence of heparin and the molecular structure of FGF1 became stabilized in the presence of heparin, we normally employed the protocol of administering the FGF1 and heparin simultaneously. However, in the case where the intestinal tracts were damaged significantly by radiation and were prone to hemorrhage, the co-administration of heparin that inhibits blood coagulation was not preferable. Therefore, the radiation damage was evaluated under the condition of not using heparin. As a result, it was shown that FGFC showed strong radioprotective action without the presence of heparin (Fig. 9 right).[15]

8.2 Protection of intestinal tract damage (treatment by post facto administration)

When using protective drugs against high-dose radiation, most cases will be administration of a protective drug after exposure (*post facto* administration). However, in reality, there are hardly any biological radioprotective drugs that produce effects in that manner of administration.

We analyzed the effect of *post facto* administration of FGFC from the aspect of protection against intestinal tract damage. FGFC was administered 24 hours after exposure to strong radiation of 10 Gy, and the growth of the intestinal crypt cells were investigated. It was shown that many cells showed growth response. This indicated that FGFC promoted growth in the few intestinal stem cells that survived the damage of radiation (Fig. 10).[15]

8.3 Protection against individual death (preventive or post facto administration)

The exposure to high-dose radiation may result in the death

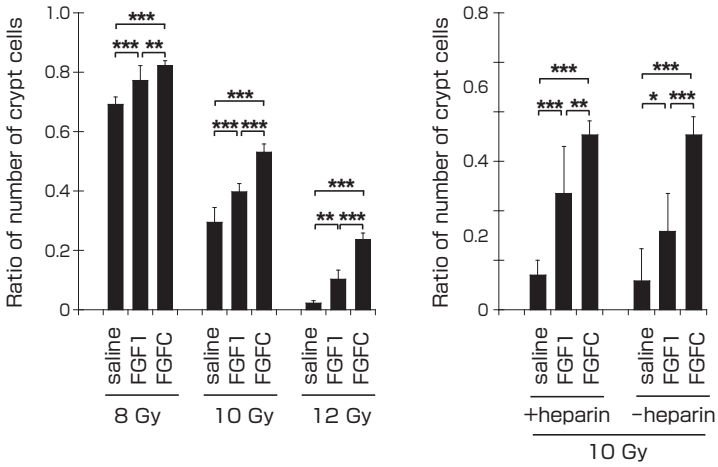


Fig. 9 Radiation damage of the intestinal tract is reduced when FGFC is administered before exposure. This activity is stronger than FGF1 in a wide range of radiation doses, and the difference becomes more apparent when heparin is not co-administered.
[Data taken partially from Nakayama *et al.*, *IJORBP* (2010).]

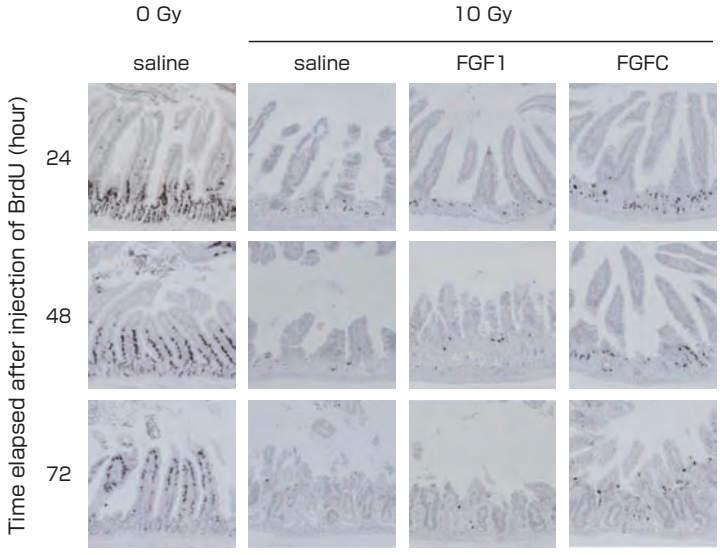


Fig. 10 The surviving cells in the intestinal epithelial stem cell niche shows proliferative response when FGFC is administered 24 hours before exposure. The photographs are cross sections of the villi that are formed by the intestinal epithelial cells. The epithelial stem cells exist in the basal part between the villi. In this experiment, the proliferating cells are stained dark brown, indicating that they are reproducing.
[Data taken partially from Nakayama *et al.*, *IJORBP* (2010).]

of the individual. Therefore, the most significant evaluation standard of radioprotective action can be considered the suppression of individual deaths. We obtained results that when FGFC alone was administered before exposure, the survival time until individual death can be significantly extended (Fig. 11).

Moreover, even when FGFC alone was administered after exposure, it was indicated that the survival time might be extended. In fact, the emergency treatment in case of high-dose exposure is not the use of radioprotective drugs alone, but multiple measures are combined. Therefore, the aforementioned life extending effect by FGFC may be further enhanced by a combination of protective drugs other than FGFC or stem cell/bone marrow transplants. Therefore, the ways of combining and the evaluation of efficacy will be future R&D topics.

8.4 Mechanism of radioprotection (preventive administration)

Then, what is the mechanism that brings about the radioprotective effect of FGFC? In general, the molecular mechanism of the action of biological radioprotective drugs has not been clarified sufficiently. First, we analyzed how apoptosis (programmed cell death) of the crypt cells was affected after radiation exposure when FGFC was administered before radiation exposure. As a result, when the two indices that indicated apoptosis were investigated, it was found that apoptosis was inhibited in the group that received preventive administration of FGFC (Fig. 12).[15]

8.5 Mechanism of radioprotection (post facto administration)

How is the protection effect expressed in the case where FGFC was administered after radiation exposure? If the exposure occurs without protection, cell death occurs and the damage is irreversible. The growth and differentiation of the intestinal epithelial cells was investigated in animals where efficacy

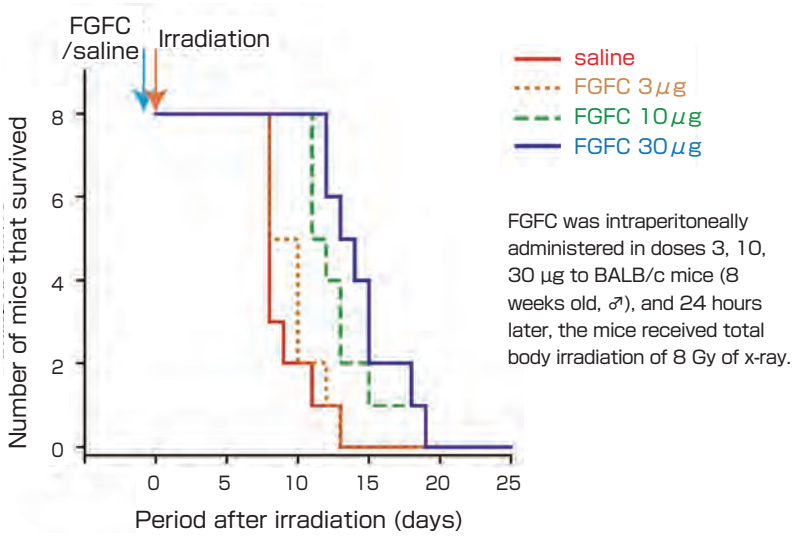


Fig. 11 The individual death after exposure is reduced and the survival period is increased when FGFC is administered before exposure.

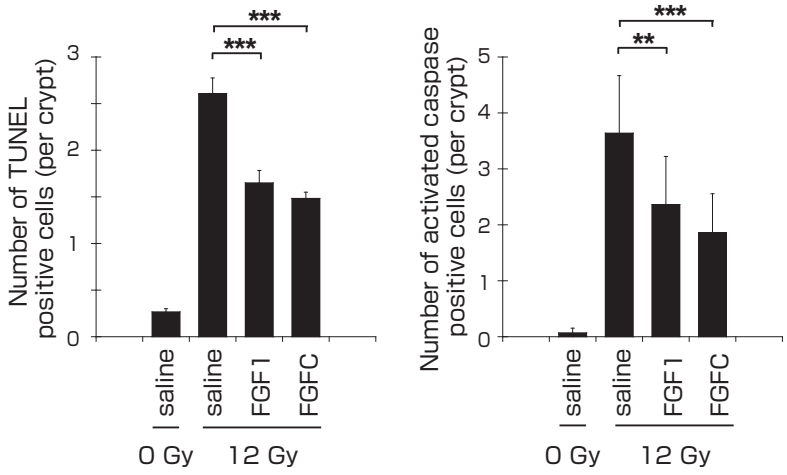


Fig. 12 It was shown that cell death was inhibited when FGFC was administered before exposure, according to both index A that indicates programmed cell death (left: TUNEL) and index B (right: activated caspase 3).
[Data taken partially from Nakayama *et al.*, *IJORBP* (2010).]

was confirmed in the *post facto* administration of FGFC after 24 hours. As a result, as mentioned in subchapter 8.2, the proliferative response of the crypt cells was confirmed. It was also shown that for the epithelial cells that possess the function of intestinal villi that occur through the growth and differentiation of the crypt cells, the expression of such growth and differentiation markers increased with the administration of FGFC (Fig. 13).[15] Therefore, it is thought that both the growth differentiation of the differentiated stem cells and the promotion of proliferation of surviving stem cells are promoted by FGFC.

Acknowledgements

In this paper, the current situation where the radioprotective drug is about to be born through the combination of the outcomes of several basic and project researches was summarized. These outcomes were attained by the works of a number of researchers. I shall list the people who directly contributed to the results described in this paper (in order of appearance in the paper; order within the item is random). Also, this research was made possible through peripheral disciplines supported by many people. I am deeply thankful to all people involved. (Honorifics abbreviated; organizations listed are those to which the researchers belonged during the research; people without organization listing belonged to the Agency of Industrial Science and Technology or AIST).

- PG-FGF1: Atsuko Yoneda, Masahiro Asada, Yuko Oda, and Keiko Ohta
- FGFC: Thomas Maciag (deceased; American Red Cross), John Anthony Thompson (Alabama University), Yoshihito Tokita, Kaori Motomura, Emi Honda, and Tadanori Tanahashi
- Protein expression system: Alan Rosenberg (Brookhaven National Laboratory)
- Cell analysis system: Masashi Suzuki, Masahiro Asada, Emi Honda, Junko Oki, Akiko Kuramochi, Yuriko Uehara, Miho Ueki, Nozomi Tsujino, Atsuko Yoneda, and David Ornitz (Washington University)
- Sendai virus vector: Mahito Nakanishi and Hiroaki Segawa
- Analysis of radioprotective effect: Masahiro Asada, Junko Oki, Megumi Goto, Akiko Hagiwara, Fumiaki Nakayama (National Institute of Radiological Sciences; Associate Researcher, AIST), Makoto Akashi (NIRS; Associate Researcher, AIST), Misawo Hachiya (NIRS), Sadako Umeda (NIRS), and Takashi Imai (NIRS; Associate Researcher, AIST)

Research Projects

1991~2000 (10 years)
Development of Core Technology for Next Generation Industry
"Development of technologies for the production and utilization of complex carbohydrates: Production of complex carbohydrates using animal cells"
2005~2009 (5 years)
R&D in the Nuclear Energy Field, MEXT
"Research for the cell growth factor for the prevention and treatment of biological damage by radiation exposure and its application technology"
2005~2008 (2 years, 10 months)
Challenging Issue Research, Signaling Molecules Research Laboratory
"Core research for signaling molecules"
2011~2012 (1 year)
Strategic Research "Development of a radioprotective drug"
2013~2015
Strategic Research "Development of technology to reduce side effects of cancer radiotherapy"

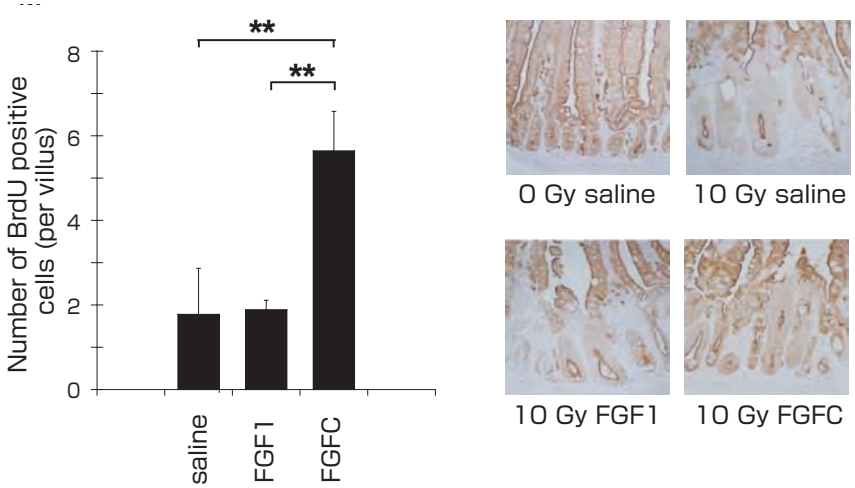


Fig. 13 It can be seen that the proliferation (left: evaluated as number of BrdU positive cells per villus) and differentiation (right: brown stain of villus differentiation marker) of the intestinal villi cells were promoted when FGFC was administered 24 hours after exposure.
[Data taken partially from Nakayama *et al.*, *IJORBP* (2010).]

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Completed the doctorate course at the Graduate School of Pharmaceutical Sciences, The University of Tokyo in 1984 (Doctor, Pharmaceutical Sciences). Joined the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry in 1984. Leader, Cell Function Laboratory, Biosignaling Department, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology in 1996; Vice Director, Gene Discovery Research Center, AIST in 2001; Vice Director, Age Dimension Research Center; Director, Signaling Molecules Research Laboratory in 2005; Vice Director, Neuroscience Research Institute; and Group Leader, Signaling Molecule RG, Biomedical Research Institute in 2010 to present. Also Visiting Researcher, American Red Cross Jerome H. Holland Laboratory for the Biomedical Sciences, USA; Adjunct Professor, Tokyo University of Science; CTO, Advangen, Inc.; and Professor, Tsukuba University. Received the Tsukuba Encouragement Award in 1996; and the Pharmaceutical Society of Japan Award for Divisional Scientific Contributions in 2013. Engages in research with focus on cell growth factor FGF for vascular endothelial cell, hepatic cell, receptor, complex carbohydrates, high order function of nervous system, muscle differentiation, dermal homeostasis and hair growth control, and protection against radiation damage.

Discussions with Reviewers

1 Title

Comment (Yoshiho Hino, Former Evaluation Department, AIST)

The title suggestion "Development of technology to reduce the biological damage by ionizing radiation" is very attractive, but I fear that it might give too much expectation to readers.

Answer (Toru Imamura)

I suggested this title from the perspective of having a simple title that can be readily accepted and understood by the readers outside the life science field. Since I received your comment that it may encompass too much, I shall use the main title and the subtitle that presents the characteristic of the scenario as follows:

Title "Development of a stable growth factor suitable for radioprotection"

Subtitle "Drug development-aimed R&D at a basic research institute"

2 Description of the scenario

Comment (Motoyuki Akamatsu, Human Technology Research Institute, AIST)

This is a paper on the scenario for sending out FGF to society as a radioprotective drug. I think the scenario and the work based on it will be useful for readers, even if the drug itself has not yet been achieved. Since the scenario for sending the general drug out to society and the scenario for a radioprotective drug are different, I think you will be able to draw the readers' attention further if you emphasize the scenario that is unique to radioprotective drugs.

Also, I get an impression that the relationship between PG-FGF1 and FGFC is unclear. If you have multiple scenarios running concurrently, please consider explaining this by using a diagram of the scenario.

Comment (Noboru Yumoto, AIST)

The objective of this research is the "development of a new radioprotective drug that possesses activity that surpasses the current drugs." Particularly for FGFC, the central focus is on the discovery of excellent properties as a drug such as "having high activity independent of heparin" and "possessing higher stability than FGF1," the elemental technologies such as "establishment of a mass production system" and "optimization of the molecular structure with an eye on pharmaceutical use" are integrated, and the protective action against intestinal tract damage and individual death is demonstrated through animal experiments. However, while PG-FGF1 is a unique molecule, there are still issues in the "quality control" and the "establishment of a mass production system" needed for drugs. Therefore, in this paper, I think you should focus on the results of FGFC, and keep the reference to the results of PG-FGF1 to a minimum if addressed at all.

Answer (Toru Imamura)

Thank you very much for understanding the significance of the research and the scenario. Based on your suggestion, I described the point that the scenarios to send products out to society differ for general pharmaceuticals and radioprotective drugs.

PG-FGF1 and FGFC are in the midst of concurrent development processes, but PG-FGF1 is way too advanced and its road to product realization is distant, and the road to product realization can be seen only for FGFC currently. Therefore, I focused mainly on the description of FGFC in this paper. However, if products from both molecules are assumed in the future, I believe PG-FGF1 will be a better product. In other words, the FGF drug that is currently available on the market can be considered first generation, FGFC will be second generation, and PG-FGF1 will be third generation.

3 Process to pharmaceutical approval

Comment (Yoshiho Hino)

In establishing the medical drug production system aiming at obtaining approval, it is needed to do the safety test and efficacy test, but this is described in such general terms, and it is not clearly or specifically described who does what.

Answer (Toru Imamura)

This research has difficult issues common to drug discovery oriented research at AIST. The substance for which drug discovery development is being done is thought to be one of the closest to exit among the intellectual properties of AIST.

To develop a drug at AIST alone all the way to the final phase is impossible in terms of funding and organization. However, even if we engage in "R&D that disregards the manner of drug discovery," it will not be a true *Type 2 Basic Research* that leads in to *Product Realization Research*. There are strict screenings before a substance with a novel effect is approved as a drug, and I think the role of *Type 2 Basic Research* in the drug discovery at AIST is to create the foundation for withstanding such screening. Here, the "safety test and efficacy test are described in general terms," but please understand that there are many development elements and difficulties in conducting such tests. Considering your indication, I eliminated a large part on the establishment of a production system and a safety test, and kept their descriptions brief.

4 Overall structure of the paper

Comment (Noboru Yumoto)

As stated in the subtitle "Drug development-aimed R&D at a basic research institute," there are three parts to the drug discovery process: 1) a part that can be done mainly at basic engineering research institute such as AIST, 2) a part that can be done collaboratively at medical institution and company, and 3) a part that can be done mainly at pharmaceutical company. In this paper, about 1) and 2), the focus is on the result, while with 3), the process is described in chapter 8. However, I think the description centering on the results makes this paper suitable for *Synthesiology*. Considering the readers who are not knowledgeable about the drug discovery process, I think you should explain in detail the overall structure of the scenario at the beginning of the paper, and make clear that you are describing the results of 1) and 2) in this paper. With the current description, the readers who started to read the paper expecting some results for a radioprotective drug, which currently is drawing a lot of attention, may end up with the impression that it is far from practical use. By clarifying the part that can be done at a basic research institute, I think you can give a positive impression that so much has been accomplished.

Also, I think whether you develop a radioprotective drug or whether you develop a drug to mitigate the side effects of cancer therapy require different scenarios. Since the description of a scenario is important for a *Synthesiology* paper, please describe from what perspective you changed the scenario.

Answer (Toru Imamura)

As you indicated, considering the readers who are not familiar with the drug discovery process, I changed the structure of the paper to carefully explain the scenario in the beginning using Fig. 2. To do so, the items were rearranged, and the positioning of this subject in *Synthesiology* was stated in chapter 1 "Introduction," the introduction to radioprotective drugs was given in chapter 2, and the scenario and the synthesis method, as well as the reason for developing the drug to mitigate the side effects of cancer therapy was explained in chapter 3.

5 Internal and external exposure

Comment (Yoshiho Hino)

The FGF drugs originally assume high-dose radiation of "external exposure," and I am not sure whether you need to mention "internal exposure." In general, for internal exposure, there is a possibility that the damage may occur due to the "cumulative effect over a long time," and the causative substance must be excreted from the body by other processes. Please describe clearly to what level of exposure this currently developed FGF drug is effective.

Answer (Toru Imamura)

I added an explanation that clarifies the range of the developed drug in Fig. 1.

For internal and external exposure, the descriptions are left as is because the biological effect will occur in the case of internal exposure by a radioactive substance that emits strong gamma rays, and the mechanism is the same as high-dose external exposure. For the damage by internal exposure, the in-depth discussion of the heterogeneity of biological damage caused by alpha and beta rays was avoided in this paper.