厚生労働科学研究費補助金（創薬基盤推進研究事業）
研究分担報告書

前立腺腫瘍、前立腺肥大組織の移植維持系の確立

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研究要旨：前立腺がんの臨床知見からマクロファージ活性が前立腺がんの進展に大きく関与することを報告し、マクロファージ機能他、異物反応の低下したSCIDマウスへの移植成功、継代維持のキーポイントになることを示した。２２年度は、前立腺がんおよび前立腺肥大症の臨床経過の指標となるＰＳＡのヒト血中濃度が研究代表者野村らの実施している移植 SCID マウス血中ＰＳＡ微量測定法の結果と対応していることを確認すると共にＰＳＡ組織染色も行った。

A. 研究目的

ヒト前立腺がんは、通常の SCID マウスやヌードマウスでは、移植後、増殖し難い。その原因について臨床的知見を得るとともに、SCID マウスへの移植、継代維持のための適切な前立腺肥大組織、がん組織を選択するとともに、前立腺がんおよび前立腺肥大症の臨床経過の指標となるＰＳＡのヒト血中濃度が研究代表者野村らの実施している移植SCIDマウス血中ＰＳＡ微量測定法の結果と対応していることを確認すると共にＰＳＡ組織染色も行い、前立腺疾患治療薬の効果と安全性に資する。

B. 研究方法

前立腺がん、前立腺肥大手術臨床例について、前立腺および周辺組織の生体内での環境（免疫、ホルモン等）を臨床医学的に調査し、前立腺がんの発生、増殖に関与する因子を究明するとともに、得られた知見をもとにSCIDマウスへの移植に適切な前立腺がん、前立腺肥大組織を選択する。

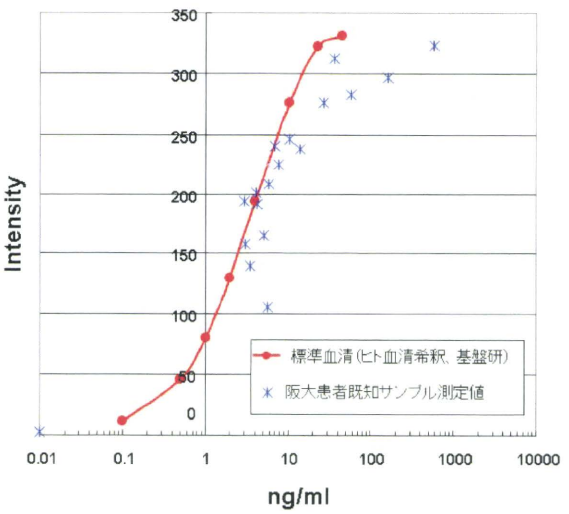
前立腺がんおよび前立腺肥大症患者の血清ＰＳＡ値測定および組織染色を行い、移植SCIDマウスの血清中ＰＳＡ値、組織染色との比較を行う。

（倫理面への配慮）ヒト前立腺組織および血清採取に当たっては、診断と治療に影響を与えない範囲での組織の利用とし、大阪大学医学部における臨床研究倫理審査委員会での承認を受けた上で採取組織の利用を行った。SCIDマウスへの移植に関しては、医薬基盤研究所において、研究総括代表者・野村が総括報告書に記載した如く倫理委員会および動物実験委員会の承認を得て移植を行っている。

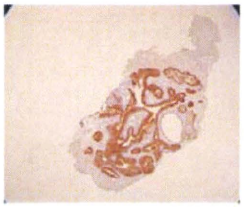
C. 研究結果

昨年度に引き続き、前立腺がん組織周囲へのマクロファージ等自然免疫関連細胞の浸潤は、ホルモン療法後の癌細胞の進展に深く関わり、予後規定因子になっている事を報告した。これらの臨床知見に基づく前腺がん症例1例と前立腺肥大症例2例の切除を行い、医薬基盤研究所において、総括研究代表者がＬＰＳ無反応の新らたなsuper SCIDマウスへ移植したところ、総括報告書に記載されているように臨床所見に合致して、前立腺がん組織は順調に増殖し、前立腺肥大組織の長期維持されている。テストステロンの付加の必要性も検討された。雌SCIDマウスには生着しなかった。

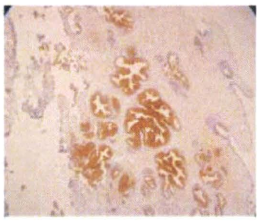
SCIDマウスへ移植した前立腺がん組織や肥大組織はSCIDマウス血中ＰＳＡ値に対応し、組織染色によってもよく発現していた。



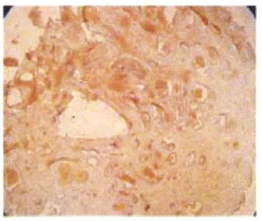
グラフは、基盤研にて、ヒトＰＳＡ標準液および患者血清を用い微量測定したものである。検量曲線（詳細は、総括研究報告書）は、0.5-50 ng 間でほぼ直線を示し、その間では患者血清値は、ほぼ同等の値を示した。移植組織のＰＳＡ組織染色にても陽性であった。



前立腺がん原発巣組織PSA染色
(Exp. 471)
患者血清PSA値：3045 ng/ml



前立腺肥大症患者原発組織PSA染色
患者血清PSA値：5.37 ng/ml



前立腺肥大症組織SCIDマウス移植4日目PSA染色(Exp. 548-D-1)

D. 考察

移植した前立腺がん組織は精巣転移症例であったため、SCIDマウスへの移植は不成功だったものと考えられる。前立腺がん、肥大症移植組織の機能も特殊SCIDマウスではよく維持されているものと思われる。

E. 結論

前立腺がんの臨床知見からマクロファージ活性が前立腺がんの進展に大きく関与することを報告し、マクロファージ機能他、異物反応の低下した総括代表者の作成した特殊なSCIDマウスへの移植により成功したものと思われる。前立腺がんおよび前立腺肥大症の臨床経過の指標となるＰＳＡが移植SCIDマウス中、移植組織でも検出され、マウス体内で前立腺がん、肥大症組織がよく機能していることを示している。

F. 健康危険情報

該当するものはない。

G. 研究発表

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2）野々村　祝夫、中井　康友、　中山　雅志、西村　和郎、　前立腺癌の発生と進展における炎症の影響　日本癌学会　第69回大会、9.22～24、大阪、2010

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4）野々村　祝夫、永原　啓、中井　康友、中山　雅志、高山　仁志、奥山　明彦、木村　泰典、中村　晃和、三神　一哉、三木　恒治、難治性精巣腫瘍に対する大量化学療法　第98回日本泌尿器科学会総会　4.27-30、岩手、2010

5）野々村　祝夫、永原　啓、辻村　晃、西村　和郎、垣本　健一、中村　晃和、三神　一哉、本郷　文弥、木村　泰典、三木　恒治、進行性精巣腫瘍に対する化学療法後の射精神経温存後腹膜リンパ節郭清術　日本癌治療学会　第48回大会、10.28～30、京都、2010.

H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　特になし

厚生労働科学研究費補助金（政策創薬総合研究推進事業）
研究分担報告書

婦人科腫瘍、胎児組織の移植維持系の確立

研究分担者　榎本隆之　大阪大学　准教授

研究要旨：卵巣癌・子宮内膜癌において、Annexin A4 が蛋白質Aを介して抗癌剤感受性に関与していることを明らかにした。また、卵巣癌細胞は抗癌剤にさらされると、細胞死を起こす前に GLUT1 が細胞膜から細胞内へ移動することによってグルコースの取り込みが減少することを明らかにした。

A. 研究目的

婦人科癌に対しては抗癌剤治療が行われることが多い。昨年度、Annexin A4 が卵巣癌の抗癌剤感受性に関わることを明らかにしたが、本年度はそのメカニズムを明らかにし、また、子宮内膜癌における Annexin A4 の役割についても解析する。さらに、卵巣癌細胞を抗癌剤処理した場合に細胞死を来たす前にグルコースの取り込みが減少することを昨年度明らかにしたが、本年度はこのメカニズムを解明し、臨床応用へ発展させる。また、卵巣癌・子宮内膜癌の発生・進展に関わる新規遺伝子･蛋白質を明らかにする。以上のことを本年度の目的とする。

B. 研究方法

1. 卵巣癌・子宮内膜癌において Annexin A4 が抗癌剤感受性に関わるメカニズムについて、細胞株および当マウスを用いて解析した。
2. 癌細胞を抗癌剤で処理した場合に起こる変化とそのメカニズムを細胞株および臨床検体を用いて解析した。

（倫理面への配慮）

研究に際し、細胞株以外に臨床検体の採取・利用も不可欠であるが、インフォームドコンセントを得たうえで、手術で摘出された組織を用いるため、患者に危害が及ぶことはなかった。また、これを応用した臨床試験については大阪大学医学部附属病院の倫理審査委員会での承認を得た。

3. 卵巣癌・子宮内膜癌の発生・進展に関わる新規遺伝子・蛋白質については、細胞株・臨床検体などを用いて解析した。

C. 研究結果

1. 卵巣癌のみならず子宮内膜癌においても Annexin A4 が抗癌剤感受性に関わっていることを同定した。特に、癌細胞がプラチナ系薬剤にさらされた場合、Annexin A4 が蛋白質Aの発現および細胞膜への局在移動を誘導することによってプラチナ抵抗性を起こすことを他に先駆けて証明した（Kim A, Enomoto T, et al. Expert Opin Ther Targets. 2010 Sep;14(9):963-971, さらに、論文準備中）。
2. 卵巣癌細胞を抗癌剤処理した場合、細胞死を来たす前にグルコースの取り込みが減少するメカニズムとして、GLUT1 の細胞膜からの細胞内への局在変化を他に先駆けて証明した（Egawa-Takata, Enomoto T et al. Cancer Sci. 2010 Oct; 101(10):2171-2178）。
3. 卵巣癌・子宮体癌の新規関連遺伝子・蛋白質として、CRABP1, CDCP1, ALDH1 を同定した。

D. 考察

卵巣癌・子宮内膜癌の治療において抗癌剤は重要であり、Annexin A4 が蛋白質Aを介して抗癌剤感受性に関与していることが明らかになった。今後、当マウスを用いて Annexin A4 および蛋白質Aを target とした分子標的治療の開発に向けた研究を行う予定である。

また、卵巣癌細胞は抗癌剤にさらされると、抗癌剤に感受性がある場合、細胞死を起こす前に GLUT1 が細胞膜から細胞内へ移動することによってグルコースの取り込みが減少することを明らかにしたが、現在これを臨床応用すべく、卵巣癌の抗癌剤投与前後でのグルコース取り込み能の変化と抗癌剤奏効性の相関を解析する臨床試験を被験者の informed consent を得た上で行っている。

また、新規卵巣癌･子宮内膜癌関連遺伝子・蛋白質である CRABP1, CDCP1, ALDH1 については、今後、当マウスを用いて、そのメカニズムの解明

と分子標的治療の開発に向けた研究を行う予定である。

E. 結論

卵巣癌・子宮内膜癌の治療において抗癌剤は重要であり、Annexin A4 が蛋白質Aを介して抗癌剤感受性に関与していることが明らかになった。

また、卵巣癌細胞は抗癌剤にさらされると、抗癌剤に感受性がある場合、細胞死を起こす前にGLUT1 が細胞膜から細胞内へ移動することによってグルコースの取り込みが減少することを明らかにしたが、現在これを臨床応用にむけた臨床試験を行っている。

F. 健康危険情報

上記臨床試験については、大阪大学医学部附属病院の倫理審査委員会での承認を得ており、それに基づいて厳格に行われている。

G. 研究発表

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2. 学会発表

H. 知的財産権の出願・登録状況
(予定を含む。)

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 特になし

III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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TAISEI NOMURA

TRANSGENERATIONAL HEALTH CONCERNS FROM RADIATION IN MICE AND HUMANS

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First, I will show one photograph before talking on "Transgenerational Health Concerns from Radiation in Mice and Humans" at the Symposium I "Environments and Health Concern from Ionizing Radiation" of the 15th Alexander Hollaender Course; Genome-Environment Interaction and Genetic Toxicology. United Nations Scientific Committee on the Effect of Atomic Radiation (UNSCEAR) meeting was held in 1958 for publishing the report on the Effect of Atomic Bomb at Hiroshima and Nagasaki and Bikini disaster by the hydrogen-bomb testing. On the way to UNSCEAR-meeting, representative Japanese scientists visited Oak Ridge National Laboratory (ORNL) on February 1-2, 1958. There were three representative scientists from Japan, Dr. Alexander Hollaender, director of Biology Division of ORNL and young Japanese scientist, Dr. Sohei Kondo who later became the professor of Osaka University. In Biology Division, Dr. W. L. Russell was continuing the very important mouse experiment on the hereditary effects of radiation, so- called "Mega-Mouse Experiment" using specific loci method.

In the symposium-1, I will show the summery of germ cell mutation conducted in ORNL and Harwell and then review my works on transgeneratinal health concerns from radiation in mice and humans.

Wild type mice were irradiated with radiation and then mated at various intervals with tester stock (T strain) of mice which have 7 recessive mutations. Seven specific loci mutations were detected at coat color loci, brown (*b*), pink-eyed dilution (*p*), chinchilla (c^{ch}), dilute *(d)*, pie bald *(s)*, non agouti *(a)* and short-ear (se). Male germ cells at spermatozoa stage was more sensitive to ionizing radiations than those at spermatogonia stage for mutation induction and 6-fold higher incidence of mutation was induced by neutron exposure than by γ -ray-exposure, showing high relative biological effectiveness (RBE) of neutrons. The most important result by specific loci method is that mutation frequency by low dose rate exposure to γ -rays is one third of that by high dose rate exposure, showing an apparent dose rate effects. Mouse oocytes showed much higher dose rate effects (~20). Furthermore, mutation frequency at low dose rate exposure increases linearly without threshold, doubling doses being 1 Gy. The value of 1.0 Gy is universally used for the genetic risk estimation of radiation to humans until now. Direct estimation with markers for human diseases such as dominant cataract and skeletal mutations were carried out and mutations were induced in mice after germ-cell exposure to radiation. These radiation-induced mutations were deletion mutations.

Mouse studies on Germ cell mutations, carried out at Oak Ridge, Harwell and Munich provided extremely important information for the risk estimation. Since enormous numbers (million) of mice were necessary, however, no more studies have been carried out until now. Instead of specific locus method for germ cell mutations, more concise method using similar tester strains of mice were carried out in a few countries. Recessive coat color mutant mice PT and HT were used in my Department. PT (T) female *(a/a, b/b, p* c^{ch}*/p* c^{ch}*, d/d)* and HT male mice *(a/a, ln/ln, pa/pa, pe/pe)* were mated, and pregnant mice were exposed to radiation on the 10.5th day of gestation. PTHTF1 embryos were heterozygous at 7 recessive coat color loci *(ln/+, pa/+, b/+, p* c^{ch}*/+ +, d/+, and pe/+)* on non agouti background (a/a). If mutation occurs at one of the several thousands of pigment

cells in the embryo, a small spot of mutant coat color appears on the black coat color back ground. From the color, size, shape, and distribution of the pigment of the hair, mutated gene loci were determined. The frequency of mutant spots increased linearly with doses of X-rays (0.1 to 1.2 Gy). With much smaller number of F1 mice (100 vs ten thousand) and much lower doses (0.1 to 1.0 Gy vs 1-10 Gy), mutations were detected more precisely by this PT-HTF1 method. Furthermore, there were no differences in mutation rate per cell between germ line specific loci mutation and in utero somatic mutations. Dose dependant increases of mutations were also observed after exposure to tritium water (3H-?-rays) and 252Cf-neutrons. Higher RBE's were shown for 3H (2.5) and 253Cf-neutrons (6.6).

Recently, in vivo and in vitro methods to detect point mutation in bacterial gene, Big Blue® TG mouse and MutaTM mouse were used for mutation assay. However, these systems are much less sensitive to radiations than PT-HTF1 method, e.g. no mutations were detectable even at lethal doses, and some false negative results were observed with chemicals, because C57BL mice were commonly used in the background.

In addition to germ cell mutations, parental exposure of mice to radiation causes a variety of adverse effects (e.g., tumors, chronic diseases, congenital malformations, genetic instability, etc.) in the progeny and the tumor-susceptibility phenotype and also some congenital anomalies are transmissible beyond the first post-radiation generation. The induced rates of tumors were 100-fold higher than those known for mouse specific locus mutations. These studies had been referred to under the headings of "Osaka Report", "Transgenerational Carcinogenesis and Teratogenesis", "Paternal Toxicology", or "Male-mediated Developmental Toxicology", although preconceptional exposure of females also induced such effects in the offspring.

An apparent and visible evidence of transgenerational effects of radiation is that exposure of male mice to radiation at post-meiotic stage resulted in a high incidence of embryonic deaths (dominant lethals) in the offspring. The frequencies increased with increasing doses of X-rays, e.g., about 60 percent dominant lethality after an X-ray dose of 5 Gy to spermatids.

In the surviving fetuses, various types of congenital malformations were observed. Most of induced malformations are those that are commonly observed in humans. Similar results were obtained in experiments in which the females were irradiated.

The incidence of tumors in F1 offspring increased with X-ray doses to the parents in the range from 0.36 to 5.04 Gy. It is evident that post-meiotic stages are more sensitive than the spermatogonial stages. No differences were observed in the incidence of tumors between single and fractionation doses after post-meiotic exposure. However, with fractionated doses given to spermatogonia, large reductions in tumor incidence in F1 offspring were observed, the figures approaching those seen in controls. Oocytes in the mature follicle stages were resistant to single dose of X-rays up to 1 Gy for tumor induction in F1, but large increases were observed at higher doses. Again, dose fractionation to mature oocytes showed large reduction in tumor incidence as was also the case with fractionated spermatogonial irradiation. With protracted irradiation of either spermatogonia or oocytes, no increase in tumor frequencies was found in F1. These observations suggest significant repair capabilities of spermatogonia and mature oocytes. Later, the results were confirmed with low dose rate exposure to 60Co -rays at the spermatogonial stage.

To confirm that the induced tumors are heritable, the F1 progeny of treated parents were mated and their progeny examined. Tumor incidence was significantly higher in the F2 and F3, when F1 or F2 male parents had tumors. The pattern of inheritance is that of a dominant with about reduced penetrance. Reduced penetrance was also found for dominant skeletal mutations. Inheritance of induced tumors was also examined in the N5 strain which develops various types of tumors. Higher incidence of tumors has been found to persist (up to F34). The pattern is similar to that of Li-Fraumeni syndrome in humans, suggesting the inheritance of tumor susceptibility.

We studied radiation-induced visible chromosomal changes cytogenetically in the offspring. However, induced germ cell changes causing tumors are not related to gross chromosomal changes detectable with the cytogenetic techniques employed, though numerical chromosomal abnormalities were present in the abnormal fetuses. The majority of tumors induced in the progeny of radiation exposed parents were commonly observed types in the respective strains of mice. In commonly observed types of tumors, we rarely found oncogene activation and p53 mutations, although germ-line alterations could produce some rare types of tumors and molecular changes. Ras, mos and/or abl genes were amplified in some rare tumors and lymphocytic leukaemia.

As discussed earlier, the important features of the presumed germ-line alterations causing tumors in the progeny of irradiated mice that we have observed are: (1) 100-fold higher incidence of tumors in the offspring than ordinary (i.e., specific locus) mouse mutations; (2) transmissible but weak carcinogenicity of radiation by itself with manifestation by postnatal environment; (3) inheritance of tumor susceptibility; (4) strain-dependent differences in background incidence of tumors. All these considered together suggested to us, early on 1978 that mutations at hyper-valiable sites or changes in gene expression of normal functional genes involved in immunological, biochemical, and physiological functions may elevate or enhance tumor incidences in each strain.

There is a considerable literature on germ line mutations induced at the mouse ESTR (expanded simple tandem repeat) loci by both low and high LET (neutrons from 252Cf) irradiation. In the work of Dubrova et al, however, spermatogonial stages were found to be more sensitive than postmeiotic stages and there were no dose rate effects for these mutations induced in spermatogonia. Their results are different from those of other investigators and are also at variance with findings from studies with specific locus mutations, and also our mouse studies.

Since changes in the expression of many genes are known to occur in tumors, we are carrying out gene expression studies to test the above hypothesis using the micro-array technology. In these experiments, the affected F1 offspring (with tumors or malformations) of N5 male mice exposed to 2.16 Gy of X-rays at spermatogonial stage were used as the starting material. These animals were mated to their litter mates as young adults and their offspring were examined for the presence or absence of tumors at twelve months of age and compared with concurrent control mouse offspring. It is clear that considerable numbers of genes are altered and such altered genes pre-exist in the tissues of cancer-prone progeny, and the majority of the abnormally expressed genes are those involved in normal physiological, biochemical and immunological functions. Consequently, changes in gene expression seem to occur in various normal functional genes rather than oncogenes per se in irradiated cancer-prone or tumor-susceptible descendants, and their progressive accumulation may contribute to cancer as we have hypothesized. Knowledge of the numbers of such oncogene-related genes and tumor suppressor-related genes has been increasing enormously during the past few years with the advance of biomedical research and technology.

Mouse studies strongly suggest that parental exposure to radiation causes various adverse effects in the progeny and these disorders transmit to further generations. However, this study has not been supported by the epidemiological study on the children of atomic bomb survivors in Hiroshima and Nagasaki who were exposed to an average dose of 435 mSv, although, in 1990, Gardner et al. reported that there was about 6-8 fold higher risk of leukemia in the children of fathers who were employed at Sellafield nuclear reprocessing plant and had been exposed to 10-100 mSv of radiation before conception and some epidemiological studies reported (but have not proven) an increase of leukemia in the children of fathers who had been exposed to low doses of diagnostic radiation. In the on-going health effects study, there is no evidence that multifactorial diseases increase in the children of atomic bomb survivors.

Induced rates of congenital anomalies in mice are compatible to those in humans, and we have to wait until children of atomic bomb survivors become cancer prone ages, i.e., adult types of

cancer develop. As for mutations, electrophoresis mutations of serum proteins (base substitution mutation) were examined in the children of atomic bomb survivors, while radiation induces deletion mutations such as specific locus mutation, dominant skeletal and cataract mutations in mice. In fact, no electrophoresis mutations were induced in the offspring of mice exposed to high doses of radiations. We have to examine mutations which are inducible by radiation.

As it was in mouse ESTR studies, 1.6~1.8 fold increases of minisatellite mutations were reported by Dubrova *et al* in the children of inhabitants in exposed areas of Chernobyl, Semipalatinsk and Techa River. Increased ratio was equal irrespective of the exposed area or populations. However, no increases of minisatellite mutations were reported by other investigators in the children of atomic bomb survivors exposed to high doses of radiation (1.9 Sv) and children of liquidators in Chernobyl (3 independent reports).

Another studies are currently underway in our laboratory using microsatellite probes to detect germ cell mutations in the offspring of ?-ray and neutron-exposed male mice. The preliminary results show that mutations at one specific microsatellite locus and also leukemias are clearly and dose-dependently induced by 0.2-0.4 Gy of neutrons in the offspring (unpublished data). It should be borne in mind, however, that no increases of microsatellite mutations have been found in the children of Chernobyl liquidators exposed to very low doses (< 50 mSv) and also in the children of atomic bomb survivors exposed to very high dose of radiation (av. 1.9 Sv) (in press). In contrast to minisatellite mutation, microsatellite germ-line mutation can be detected precisely even with one base change and with much less numbers of animals than ordinary mouse germ-line mutations. We believe that mutation studies with microsatellite are useful to detect transgenerational effects or genomic instability by environmental toxic chemical substances as well as radiations, but we have to find human gene loci which are responsible to radiation or chemicals for mutation induction. This technology is also very useful to examine the hereditary effects of space radiations, because several hundreds offspring are produced from 5-10 male mice which are launched into the space.

E. Tajima, M. Tochiku, and Y. Hiyama (from the left end to right) from Japan, Alexander Hollaender, director of Biology Division of ORNL (right end) and young Japanese scientist, Sohei Kondo (2nd from the right end). Provided by Professor S. Kondo

Minisatellite Mutations in Radiation Exposed Human Population

		Exposed	Control	
Atomic Bomb Survivors (Japan)	No change	62 children (1.9 Sv)	60 children (<0.01Gy)	Kodaira, et al (1995)
Chernobyl (Belarus) (Inhabitant)	Increase(x1.8)	127 children	120 UK non-exposed	Dubrova, et al (1996)
Atomic Bomb Survivors (Japan)	No change	61 children (1.9 Sv)	58 children (<0.01Gy)	Kodaira, et al (2004)
Chernobyl (Ukraine) (Liquidator)	No change	183 children	163 children Non-contamn. area	Livshits, et al (2001)
Chernobyl (Ukraine) (Inhabitant)	Increase(x1.6)	240 children	98 matched control	Dubrova, et al (2002)
Chernobyl (Estonia) (Liquidator)	No change	148 children	155 children born before exposure	Kiuru, et al (2003)
Chernobyl (Ukraine) (Liquidator)	No change	51 children	24 children born before exposure	Slebos, et al (2004)
Semipalatinsk (Kazakhstan) (Inhabitant)	Increase(x1.7)	40 families (3 generations)	28 families (3 generat) (matched control)	Dubrova, et al (2002)
Techa River (Mayak) (Russia) (Inhabitant)	Increase(x1.7)	159 children (50 mSv)	110 children (non-contamn. area)	Dubrova, et al (2006)

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Excretion into feces of asialo GM1 in the murine digestive tract and *Lactobacillus johnsonii* exhibiting binding ability toward asialo GM1. A possible role of epithelial glycolipids in the discharge of intestinal bacteria

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Abstract In the digestive tract of mice (HR-1, 5 months old, ♀), asialo GM1 (GA1) exhibiting receptor activity toward several intestinal bacteria was preferentially expressed in the small intestine. Also, less than 10% of GA1 in the small intestine was converted into fucosylated and sulfated derivatives, but it was completely converted to fucosyl GA1 (FGA1) in the stomach, cecum and colon. Among the lipid components in these tissues, glycolipids other than Forssman antigen and cholesterol sulfate (CS) were present in the digestive tract contents. However, sulfated GA1, sulfatide and fucosyl GM1 in the gastrointestinal contents were not present in the cecal and colonic contents, in which the major glycolipids were ceramide monohexoside (CMH), GA1 and FGA1. The total amount of GA1 in the whole contents was 20% of that in the tissues. Thus, glycolipids were stable during the process of digestion, and excreted from the body together with cholesterol and CS. On the other hand, *Lactobacillus johnsonii* (LJ), whose receptor is GA1, was detected in the cecal and colonic contents on sequential analysis of 16S-ribosomal RNA and TLC-immunostaining of antigenic glycolipids with anti-LJ antiserum. LJ was found to comprise 20% of the total bacteria cultured in the lactobacillus medium under aerobic conditions, and to be present in the cecal and colonic contents, 9.8×10^7 cells versus 37 μg GA1 and 1.4×10^8 cells versus 49 μg GA1, respectively. Thus, GA1 in the contents might facilitate the discharge of intestinal bacteria by becoming attached them to prevent their irregular diffusion.

Keywords Sphingoglycolipids · Glyceroglycolipids · Molecular species · Lactobacillus · 16S-rRNA · TLC-immunostaining

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Abbreviations

The nomenclature for glycolipids is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [1]

CMH	ceramide monohexoside
CS	cholesterol sulfate
18t:0	phytosphingosine
18d:1	sphingosine
24h:0	α-hydroxylignoceric acid
GA1	asialo GM1
FGA1	fucosyl GA1
FGM1	fucosyl GM1
SGA1	sulfated GA1
LJ	*Lactobacillus johnsonii*

Introduction

Asialo GM1 (GA1, Gg_4Cer) is known to constitute the receptor for several bacteria, *i.e. Lactobacillus casei, L. reuteri, L. johnsonii (L. acidophilus), Bifidobacterium*

bifidum, Pseudomonas aeruginosa, Actinomyces maeslundii and Neisseria gonorrhoeae, and to play an essential role in bacteria in the establishment of symbiosis or infection [2–4]. In the murine digestive tract, GA1 is abundant in the small intestine, but not in the stomach or colon, suggesting that the small intestine is the site of GA1-mediated formation of the bacterial flora for symbiosis [5]. However, although GA1 is a predominant glycolipid in the small intestine of germ-free mice, fucosyl GA1 (FGA1, $IV^2Fuc\alpha$-Gg_4Cer) is present in the small intestine of conventionally breeding mice [6]. The modification of GA1 to FGA1 has been revealed to occur on transcriptional induction of the α1,2-fucosyltransferase gene on infection by indigenous filamentous bacteria and wild type *Bacteroides thetaiotaomicron*, indicating that some bacteria regulate gene expression in the intestinal epithelial cells of the hosts, probably to suppress the growth of competitive bacteria [6–8]. Consequently, the flora formation through attachment to GA1 of symbiotic bacteria such as *Lactobacillus johnsonii* (LJ) is dynamically regulated through modification of GA1 by the coexisting bacteria [3, 4]. In addition, the expression of GA1 and FGA1 in the intestinal microvilli occurs as an event of the differentiation process during the migration of epithelial cells from the crypt to the top of villus [9], and the cells accompanying the bacterial flora are thought to be finally liberated into the digestive tract.

In the literature, comparison of glycolipids in the gastrointestinal tract and feces of germ-free and conventional rats demonstrated that feces contained intestinal glycolipids with ABO blood groups, and the amount of LacCer as the major glycolipid in the feces of conventional rats were higher than in germ-free mice [10]. Similarly, human intestinal glycolipids have been shown to be excreted into the feces, in which GlcCer, GM3 and Le^a, and GlcCer and LacCer were the major ones before and after the weaning period, respectively, and the reason why LacCer becomes the major glycolipid in human feces after the weaning period is the degradation by glycosidases produced by bacteria [11, 12]. However, since these studies were focused on structural characterization of glycolipids in feces, and not on quantitative analysis, the rates of excretion and degradation of epithelial glycolipids were unknown. Also, no detailed information on glycolipids in the contents of different regions of the murine digestive tract in comparison to those in the tissues and murine food is available so far.

Accordingly, we determined the total amounts and concentrations of glycolipids in the tissues and contents of various regions of the murine digestive tract, in which glycolipids were expressed in region-specific manners [5]. The information was thought to be useful for estimating not only the rate of turnover of epithelial glycolipids, but also the amounts of receptor glycolipids excreted together with bacterial flora. In this connection, we also determined the amounts of *Lactobacillus johnsonii*, whose receptor glycolipid is GA1 [2, 3], in the cecal and colonic contents through direct detection of bacterial glycolipids in lipid extracts of the contents by means of a newly developed method of TLC-immunostaining involving anti-LJ antiserum.

Materials and methods

Bacteria

The bacteria used in this study were purchased from the Japan Collection of Microorganisms (JCM, RIKEN, Wako, Saitama, Japan), and were as follows: *Lactobacillus johnsonii* (LJ) (JCM 1022) and *L. casei* (JCM 1134). The culture media for bacteria were as follows: MRS broth (Gibco-Invitrogen) and GYP (1 g glucose, 1 g yeast extract, 0.5 g peptone, 0.2 g meat extract, 0.2 g sodium acetate, 20 mg $MgSO_4$, 1 mg $MnSO_4$, 1 mg $FeSO_4$, 1 mg NaCl and 50 mg Tween 80 in 100 ml water). The number of lactobacilli was determined from the colony formation on a $CaCO_3$-containing agar-GYP plate (0.5% $CaCO_3$ and 1.2% agar in GYP medium) [13].

Mice

Mice (HR-1, female, 5 months old of age) were kept under conventional breeding conditions with lighting from 6:00 to 18:00 at a room temperature of 24±1°C and a humidity level of 55±10% with food (MF, Oriental Yeast, Tokyo) and water *ad lib*. Animal treatment followed the animal care guidelines of Kinki University. After anesthesia with pentobarbital (Abbott, Osaka), the tissues and contents from eight mice were collected separately. The upper and lower halves of the tract between the duodenum and cecum were used as the jejunum and ileum, respectively, whose contents were collected by injecting water with a syringe. The solid contents in stomach, cecum and colon were collected with a spatula. Also, a part of the cecal contents corresponding to 0.01–1 μg, was suspended in phosphate-buffered saline (PBS) and cultured on $CaCO_3$-agar GYP plates. The individual colonies on the plates were each picked up with a toothpick and then cultured in 80 ml of GYP medium.

Materials

Standard glycolipids from various sources were purified in our laboratory: GlcCer, LacCer, Gb_3Cer, Gb_4Cer and GM3 from human erythrocytes, Forssman glycolipid from equine kidney, and fucosyl GM1 (FGM1) from bovine thyroid. GA1 and FGA1 were prepared from GM1 and FGM1, respectively, by treatment with *Arthrobacter ureafaciens*