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H. 知的財産所有権の出願・登録状況（予定も含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

内分泌撹乱物質の神経行動学的評価とその脳内機序の解明

分担研究者：栗生修司　九州工業大学大学院生命体工学研究科　教授

研究要旨
ビスフェノールA(BPA)の周産期曝露での中枢影響を行動学的、組織学的、電気生理学的に調べた。トリブチルスズおよび 1-ブロモプロパン(1-BP)の行動影響も調べた。BPA およびトリブチルスズ 9μg/kg/day の曝露で探索行動および青斑核の性分化が障害された。BPA は扁桃体ニューロンのニオイ応答選択性を障害し、この領域の神経回路発達に影響を及ぼした可能性がある。また、うつ反応亢進以外に、捕食者のニオイに対する警戒反応も亢進し、外的環境に対するストレス脆弱性が示唆された。1-BP 胎生期曝露実験では、700ppm 曝露で新たに雌の性行動障害が見出され、400ppm でも、探索行動の性分化障害が認められた。

A. 研究目的

BPA および内分泌撹乱候補物質であるトリブチルスズや1-BP の中枢神経系に及ぼす影響について、濃度をさらに低くして検討した。BPA の性的二型行動、性的二型核の性分化への影響についてヨーロッパ(EU)の基準値以下で検討し、またニオイに対する警戒反応、神経活動への影響を調べた。さらに 1-BP の性行動に与える影響を調べ、曝露濃度をさらに下げた条件での性的二型行動への影響も評価した。

B. 研究方法

BPA(9μg/kg/day)，1-BP(400ppm)まで曝露濃度を落とし、その中枢影響を行動学的、組織学的、電気生理学的に調べた。

今回新たに 9μg/kg/day の BPA を周産期曝露し、オープンフィールド試験での性的二型行動および青斑核の性分化に与える影響を評価した。胎児期曝露実験も継続し、今回は捕食者のニオイに対する警戒行動を調べ、また、扁桃体内側核領域の神経活動をニオイ選択性の特性について、新たな解析を試みた。1-BP 胎生期曝露実験(700ppm)についても継続し、新たに性行動への影響を調べた。さらに曝露濃度を 400ppm まで落とし、700ppm 曝露で明らかになった性的二型行動へも影響を調べた。

(倫理面への配慮)

所属施設である九州工業大学大学院生命体工学研究科における動物実験に関する指針の規制に基づいて行った。

C. 研究結果

BPAおよびトリブチルスズの9μg/kg/day曝露でも、探索行動、青斑核の性差が消失した。胎生期曝露により、捕食者のニオイに対する警戒反応、神経活動のニオイ選択性も修飾された。1-BP(700ppm)で新たに雌の性行動障害が認められ、400ppm曝露では探索行動の性差が消失した。

探索行動の指標となるオープンフィールド試験における立ち上がり行動の回数は、活動性などと比べて鋭敏であり、今回BPAの曝露量を9μg/kg/dayまで落としても性差が消失した。特に雄への影響が強く、結果的に雌に近づき、性差が消失した。このパターンは従来の周産期(30μg/kg/day)、胎生期(15μg/kg/day)、新生児期(24μg/kg/day)における各曝露実験でも同様に確認されている。

胎生期曝露ラットで捕食者のニオイ存在下で特に雄の曝露群において、活動性低下、ニオイ回避傾向の増強が確認された。扁桃体内側核領域のニューロンについて、捕食者と非捕食者のニオイ選択性を分析した結果、雄で高選択性を示したが、BPA曝露で減弱した。

1-BP胎生期曝露実験では、700ppm曝露で新たに雌の性行動の障害(ear wiggling低下、rejection score増加)が確認された。さらに曝露濃度を400ppmまで落として、オープンフィールド試験を実施した。その結果、立ち上がり行動の性差消失が依然として見られた。1-BPの場合、雄はあまり影響を受けず、雌の値が下がって雄に近づくことがBPAと異なる。

D. 考察

EUのBPAの基準は日米よりも厳しく、10μg/kg/dayと定めている。今回の結果は、この基準値以下でもBPAは脳と行動の性分化を障害することを示したものである。前回、BPAがうつ反応を亢進させていることは示したが、今回新たに捕食者のニオイに対する警戒反応も亢進した。セロトニン系障害モデル動物は、うつが亢進し捕食者のニオイに対して脆弱であるとの報告もあり、うつ情動発現との共通の背景が示唆される。扁桃体領域の興奮性神経活動が捕食者と非捕食者のニオイに対し、雄で高選択性を示し、これは鋤鼻系を含めた嗅覚経路の性差を反映したものと考えられた。雄の高選択性がBPA曝露により減弱したのは、この領域に高発現している性ホルモン受容体を介したBPAの作用が考えられた。

1-BP実験は、今回新たに雌の性行動障害が確認され、これらのラットは2日齢における肛門生殖器間距離が雌で有意に増加したことも明らかになっている。400ppm曝露でも探索行動の性差が消失し、特に雌の値が下がり雄に近づいた。これらのことから、1-BPにはエストロゲン受容体に促進的に作用し、雌ラットをオス化させるような、内分泌撹乱物質に類似した作用があることが示唆された。

BPAが影響を及ぼした、性的二型行動、性的二型核、うつ情動、扁桃体神経回路は基本的に性ホルモン依存的に発達すると考えられている。BPAが性ホルモン受容体を介してこれらの神経機構の発達を妨げた可能性は高い。今後は、性ホルモン受容体が関連

する遺伝子を中心にしたDNAアレイによる遺伝子解析を実施したい。1-BPも、明らかになっているGABA受容体機能への作用面からのメカニズム考察は行っているが、本当に内分泌撹乱物質であるかは、今後、大幅に曝露濃度を下げた条件での検討が必要と考えられる。

E. 結論

BPAと1-BPは曝露量を下げても従来同様の影響を及ぼし、性ホルモン受容体を介した多彩な作用が考えられた。

BPAは、環境ストレス脆弱性を亢進させ、扁桃体領域の神経活動のニオイ選択性にも影響を与える。今回新たにEUの基準値以下で曝露したが、従来同様、脳と行動の性分化を障害した。1-BPは性行動を障害し、曝露量を半分程度に落としても探索行動の性分化を障害した。内分泌撹乱物質として振る舞っている可能性は高い。

F. 健康危惧情報

なし

G. 研究発表

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H. 知的所有権の取得状況

1. 特許所得　なし
2. 実用新案登録　なし
3. その他(データベースなど)　なし

厚生労働科学研究費補助金（化学物質リスク研究事業）

平成18年度　分担研究報告書

研究課題名=[マイクロアレイ基盤整備]

遺伝子発現の網羅的検索とインフォマティクスの確立

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研究要旨

本研究は、当研究班での幅広い研究対象にDNAマイクロアレイ解析技術を適用することで、他の研究機関では得られないホルモン作用メカニズムを同定することを目指すものである。そのために、我々が開発したPercellome手法を適用した網羅的遺伝子発現解析による各班員の研究サポートを実施する。

今年度実施中のサポート研究は、マウス前立腺におけるBPA作用の網羅的遺伝子発現変動解析、及び破骨細胞特異的ERαノックアウトマウス骨髄細胞の網羅的遺伝子発現解析である。

A. 研究目的

本研究の目的は、当研究班での幅広い研究対象にDNAマイクロアレイ解析技術を適用することで、他の研究機関では得られないホルモン作用メカニズムを同定することである。

すなわち、

ホルモン受容体はリガンド依存的転写因子として特定の遺伝子（群）を発現させ、胎生期には形態形成プログラムをも制御する。この遺伝子発現カスケードは、臓器ごとにその発生・発達段階により多種多様であると考えられる。例えば、ホルモン活性物質の in vivo 試験として従来から行われている子宮肥大試験や Hershberger 試験における比較的単純な endpoint でさえ、幾重かの反応カスケードの結果であると考えられる。このカスケードの解析は容易ではないが、進歩の著しい DNA マイクロアレイ技術を導入することにより包括的かつ迅速な検討を行う方途が開けてきた。そこで本研究では、当班へDNAマイクロアレイ技術を導入し、各班員が実施中の研究を網羅的遺伝子発現解析という側面からサポートする体制を整えることを目的とする。

B. 研究方法

各班員との協議の下、共同研究を行い、組織もしくは細胞の検体の供与を受け、DNA マイクロアレイ解析データ解析結果を班員にフィードバックすることで研究をサポートする。

すなわち、

各班員からの検体の RNA の分離精製

生体組織もしくは培養細胞を材料とする。生体

組織の場合は、採取後すみやかに RNAlater (Ambion 社)に浸漬し、RNase を不活化する。その際、組織の厚さが 5mm 以下となるように細切する。その後、RNA 抽出操作までは-80℃にて保存する。抽出に当たっては、RNAlater を除いた後、RNeasy キット（キアゲン社）に添付される RLT buffer を添加し、ジルコニアビーズを用いて破砕液を調製し、破砕液の 10uL を取り、DNA 定量蛍光試薬 Picogreen を用いて DNA 含量を測定する。DNA 含量に応じ、Spike cocktail(Bacillus 由来の RNA 5 種類を濃度を変えてあらかじめ混合した溶液)を添加し、TRIZOL により水層を得、RNeasy キットを用いて全RNAを抽出する。一部を電気泳動しRNAの純度および分解の有無を検討した。培養細胞の場合は、細胞に直接 RLT buffer を添加し、細胞破砕液を調製する。21G の注射針を通し、ゲノム DNA を裁断した後、破砕液の 10uL を取り、DNA 定量蛍光試薬 Picogreen を用いて DNA 含量を測定する。以降は生体組織の場合と同様である。以上のステップのうち、生体組織分離と RNA later への浸漬まで、もしくは培養細胞 RLT 破砕液調製までを共同研究先の班員が実施し、その後のステップを当方で実施した。

Genechip 解析

全 RNA 5 μg を取り、アフィメトリクス社のプロトコールに従い、T7 プロモーターの付加したオリゴ dT プライマーを用い逆転写し cDNA を調製し、得た cDNA をもとに第二鎖を合成し、二本鎖 DNA とした。次に T7 RNA ポリメラーゼ（ENZO 社キット）を用い、ビオチン化 UTP, CTP を共存させつつ cRNA を合成した。cRNA は Affymetrix 社キットにて精製後、300-500bp となるよう断片化し、Genechip ターゲット液とした。Genechip には Mouse Genome 430 2.0（マウス）、Human Genome 133 2.0（ヒト）を用いた。ハイブリダイゼーションは 45℃にて 18 時間行い、バッファーによる洗浄後、phycoerythrin (PE)ラベルストレプトアビジンにて染色し、専用スキャナーでスキャンしてデータを得た。データは当方で開発したソフトウエアとマイクロソフト社エクセルを併用して解析した。

C. 研究結果

本年度は、杉村芳樹班員とのマウス前立腺における BPA の作用の網羅的遺伝子発現変動解析および、加藤茂明班員と破骨細胞特異的 ERα ノックアウトマウス骨髄細胞の網羅的遺伝子発現解析を実施した。結果は各班員と共同で解析中である。

すなわち、

マウス前立腺における BPA の作用の網羅的遺伝子発現変動解析（杉村班員）

マウス前立腺における内分泌撹乱化学物質の影響について、Percellome 手法を適用した Affymetrix 社の Genechip システムによる網羅的遺伝子発現解析を実施した。具体的には、杉村班員より送られた細胞サンプルから、RNA 抽出を始めとする Genechip システム解析に必要な反応を行い、データを得、得られたデータを杉村班員と共同で解析中である。以下でその状況について説明する。

共同研究概要：内分泌かく乱化学物質の前立腺に対する影響の解析を目的とする共同研究を開始した。杉村班員は BPA がマウス前立腺においてエストロゲン様作用を示すことを無血清器官培

養法によって確認し、さらにBPAによりTGFaが発現誘導されることを見いだしている。しかし、BPAがTGFaのみを誘導するとは考えられず、網羅的な解析を進めることにより他の因子を検索することが今後の研究方針を立てる上で重要であると考えられた。そこで、具体的な共同研究として、BPA経胎盤投与による出生前後の前立腺における遺伝子発現変化の網羅的解析を実施した。胎生13日から16日までBPAまたはDESを経胎盤投与（母親に経口投与）し、胎生17日、18日、生直後にマウス泌尿生殖洞 (UGS)を採取した。BPA、DESの投与量はそれぞれ20ug/kg、0.2ug/kgとした。Percellome法を適用し、コピー数に変換したデータを用いて網羅的に遺伝子発現変化を解析した。その結果、ケラチン化に関わる遺伝子群（Hrnr, Ivl, Krt1-10, Krt2-1, Krt2-7, Krtdap, Lor, Sprr1a, Sprr2d, Sprr2h）がBPAで発現上昇していた。これはすでに杉村班員が見出しているBPA, DES投与に伴う前立腺の扁平上皮化生に符合する発現変化であると考えられる。また、抗ミュラー管ホルモンの発現を制御する転写因子Steroidogenic factor-1 (SF1)の発現が、BPA投与により顕著に発現上昇することも見出された。現在、この意義について杉村班員と協議中である。

破骨細胞特異的ERαノックアウトマウス骨髄細胞の網羅的遺伝子発現解析（加藤班員）

この共同研究は、エストロゲンによる骨代謝制御機構に関する研究として実施した。閉経後骨粗鬆症の原因はエストロゲン欠乏に起因する事は明らかであるとされているが、その作用機序は不明である。骨代謝に直接関わる骨芽細胞（骨形成細胞）、破骨細胞（骨吸収細胞）に対する作用が有力視されているが、全身性エストロゲン受容体(ER)ノックアウトマウスは内分泌系の異常により、骨量減少すら起きない事が分かっている。このような背景のもと、加藤班員が破骨細胞特異的なERノックアウトマウスを作製したところ、明確な骨量減少が観察され、骨に対するエストロゲン作用が強く抑制されている事から、破骨細胞が骨組織に置けるエストロゲンの主要な標的細胞であると加藤班員は結論づけている。

そこで、破骨細胞でのエストロゲン標的遺伝子を特定するために、加藤班員と協同研究を実施した。12週齢のコントロール群・破骨細胞特異的ERKOマウス群、それぞれの群に対する卵巣摘出モデル群、更にそれぞれの卵巣摘出モデルにエストロゲンを投与した群の合計6群を作製し、遺伝子発現の変動をエストロゲン投与後4時間で検討した。その結果、エストロゲン投与でアポトーシス関連遺伝子群、破骨細胞分化遺伝子群が発現上昇するのに対し、破骨細胞特異的ERαノックアウトマウスでは抑制されていた。この結果は、エストロゲンの破骨細胞に対する作用はアポトーシスの誘導であるという加藤班員のモデルに合致するものである。

D. 考察

杉村班員との共同研究で、BPAによるケラチン化とSF-1発現上昇を介した抗ミュラー管ホルモンシグナル系の活性化の可能性が見出された。ケラチン化はこれまでの研究を裏付けるものであるが、SF-1発現上昇は本研究によって初めて見出された現象である。この成果はBPAの前立腺発達に対する影響を解析する上で有用な情報となる

と考えられる。加藤班員と行った共同研究においては、エストロゲンの骨代謝制御機構を解明する一助となる成果が得られた。すなわち、破骨細胞特異的 ERαノックアウトマウスを用いることで初めて見出された ERαを介した破骨細胞抑制現象を説明する機構として、エストロゲンが破骨細胞にアポトーシスを誘導するという加藤班員のモデルを支持する遺伝子発現パターンが得られている。この成果は今後内分泌撹乱化学物質の骨代謝への影響を解析する際に貴重な情報となる。

E. 結論

本研究は、網羅的遺伝子発現解析を用いることで、各班員の研究方向決定に影響を与える情報を短期間のうちに得ることを狙いとして実施し、今年度の共同研究でもその目的を果たす成果が得られた。

網羅的遺伝子発現解析技術は、数万のマーカーを迅速に検討できる有効な技術である。本研究で示されてきたように、この技術は、既知の情報から推測することが困難な新たな情報を提供してくれる可能性を秘めた解析手法であり、明確な表現型を伴って影響が現れることが少ない内分泌かく乱化学物質の作用を、その作用メカニズムに立ち入って解析する際に今後も有効活用していくことが望ましい。

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3. 知的所有権の取得状況

A. 特許取得

なし

B. 実用新案登録

なし

C. その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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EXPERIMENTAL HEMATOLOGY

Experimental Hematology 34 (2006) 274–283

Caloric restriction prevents radiation-induced myeloid leukemia in C3H/HeMs mice and inversely increases incidence of tumor-free death: implications in changes in number of hemopoietic progenitor cells

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Objectives. **Previously, we found a clear decrease in the incidence of radiation-induced myeloid leukemia in C3H/HeMs mouse caused by caloric restriction (CalR). In this report, CalR before and after irradiation was examined to determine whether they exert different effects on the prevention of radiation-induced myeloid leukemogenesis and the consequent extension of life span by CalR.**

Methods. **The C3H/HeMS strain, which is prone to radiation-induced myeloid leukemia, was used. Groups subjected to different CalR timings, pre- and postirradiation, were compared with groups not subjected to CalR during their lifetime for the incidences of neoplasms, specifically that of myeloid leukemia, and the incidence of tumor-free death. A single dose of 3Gy X-ray was administered to mice at 10 weeks old. Results of colonization assay before and after CalR were compared with the incidence of leukemogenesis among the groups.**

Results. **Irrespective of the CalR timing in terms of irradiation, there was a significant difference in the prevention of myeloid leukemogenesis, and a consequent difference in longevity (731 ~ 805 days for CalR groups vs. 697 days for the group without CalR; Log rank, $P<0.03$). During CalR, the number of hemopoietic progenitor cells (HPCs), potential leukemogenic targets, significantly decreased (0.4×10^4 vs. 4.2×10^4 of granulomacrophage colonyforming units per spleen; 1.3×10^4 vs. 7.6×10^4 of the splenic colony forming units per spleen), but this decreased number of HPCs returned to that of the non-CalR control group, when the CalR group was returned to nonrestricted diet (returned to 1.5×10^4 granulomacrophage colony-forming units per spleen; returned to 2.8×10^4 splenic colony-forming units per spleen). Although preirradiation CalR followed by a conventional non-CalR diet negates the potential preventive effect, prevention conferred by pre-and postirradiation CalR suggests different underlying mechanisms; preirradiation CalR prevents the initiation of direct genotoxic leukemogenesis, while postirradiation CalR the indirect, epigenetic, leukemogenesis.**

Conclusion. **The incidences of tumor-free death significantly increased in all the groups undergoing CalR except for the group subjected to preirradiation CalR, which contributed to the longevity of the groups undergoing CalR.** **© 2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

Radiation-induced leukemia was noted as the highest risk factor for mortality among atomic bomb survivors in Hiroshima and Nagasaki [1,2]. Relative risk of leukemia has been estimated to be approximately 6.5, whereas that for other tumors is 1.2 [2]. Experimentally, caloric restriction (CalR) has been found to be only a preventive factor for the risk comparable to epidemiological relevancy in atomic bomb survivors. Thus, timing of restriction seems to be an additional factor that should be taken into account when trying to understand not only the underlying mechanism, but also the epidemiological relevancy of CalR.

Our previous study of CalR using C3H/He mice, which are prone to radiation-induced myeloid leukemia [3], in relation to radiation-induced leukemogenesis showed that CalR led to a significant decrease in the incidence of

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myeloid leukemias [4]. Furthermore, when timing of CalR between lifetime CalR and postirradiation CalR were compared, onset of myeloid leukemia was significantly delayed in the former compared with the latter, although both resulted in a significant decrease in total incidence of myeloid leukemias. Thus, the present study was designed to elucidate the role of different CalR timings, including preirradiation CalR, in leukemogenic prevention. Possible target cells for radiation leukemogenesis are hemopoietic stem cells, that is, long-term repopulating stem cells [5] and the population of such hemopoietic stem cells changes proportionally in response to different types of progenitor cell [6], such as granulocyte macrophage colony-forming units (CFU-GM) and other progenitors, including splenic colony-forming units (CFU-S) [7,8]. In relation to these, the number of hemopoietic progenitor cells (HPCs), and the kinetics of HPCs, i.e., cell-cycle parameters, were evaluated and compared among the CalR groups as possible markers predict leukemogenesis.

CalR induces a notable decrease in splenic weight and, consequently, in the number of HPCs, which may respond proportionally to the number of hemopoietic stem cells, the potential target cells for myeloid leukemogenesis [9]. In our previous experiments, we observed the effect of CalR throughout the lifespan of mice, which raised the question as to whether risk of leukemogenesis is a function of the number of potential target cells and, consequently, a function of the number of HPCs at the time of irradiation. In the present study, to answer this question, CalR in mice was started at 6 weeks old for the first group until the time of irradiation, at 10 weeks old, and mice were then returned to a regular non-CalR diet. In the other group, restriction was started at 10 weeks old and continued throughout their lifespan. The former treatment was designed to modify the stage of leukemogenesis before irradiation, and the latter to determine the effect of diet on the stage of leukemogenesis after irradiation. We refer to the former treatment as modification of the "initiation stage" of leukemogenesis, because this treatment modifies the number of possible target cells for leukemic initiation; and the latter stage as modification of the "promotion stage" of leukemogenesis, because this treatment modifies proliferation and differentiation of potentially initiated cells after irradiation.

CalR neither more significantly prevented radiation-induced development of neoplasms other than myeloid leukemias nor inversely increased the incidence of any neoplasm. Consequently, because of decreased incidence of myeloid leukemias, incidence of tumor-free death increased.

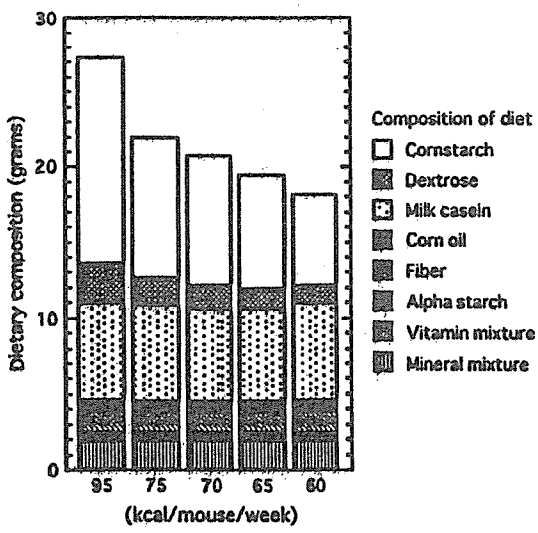

Figure 1. Five dietary regimens based on diets of different caloric contents (see text). The total, in grams, fed to each mouse per week is indicated in the bar graph for each dietary regimen. For caloric restriction, 60-, 65-, 70-, 75-, and 95-kcal dietary regimens, were used to maintain the body weight of each mouse within 25–27 g.

Materials and methods

Mice

C3H/He mice, which are prone to radiation-induced myeloid leukemia, were used in the present study. Incidence of spontaneous myeloid leukemia in C3H/He male mice is 1%, which increased to 23.3% after 3-Gy whole-body x-ray irradiation [3]. Six-week-old male C3H/HeNirsMs mice bred at our institute and released as cohort were used. Mice were housed individually, but were housed in groups if their weights were within 6% to 8% of each other, in environmentally controlled clean conventional rooms supplied with high-efficiency particulate air under a 12-hour light to 12-hour dark cycle in an authorized animal facility of the Laboratory Animal Research Center at the National Institute of Radiological Sciences. Mice were monitored weekly for maintenance of body weight, and their health status was assessed twice daily [4]. All equipment and supplies, including cages, water bottles, and wooden chips used for bedding, were sterilized.

Diets

Diets of different caloric contents, 3.31, 3.35, 3.38, 3.42, and 3.48 kcal/g, were used. Caloric intake was adjusted by varying amounts of carbohydrate and dextrose, while providing constant amounts of other nutrients, such as proteins, lipids, vitamins, and minerals (Fig. 1). Noncaloric restricted (control) mice were provided 95 kcal/week, per mouse, based on the 3.48 kcal/g diet. For CalR, according to the body weight monitored three times a week, diets were of different calorie-controlled regimens, i.e., 60, 65, 70, 75, and 95 kcal/week, per mouse (see section, Calorie Restriction Procedure).

Irradiation

Mice were exposed to 3-Gy whole-body x-ray irradiation at a 200-kV/20-mA pulse using a therapeutic x-ray irradiator (Simadzu, Kyoto, Japan) with 0.5-mm Al and 0.5-mm Cu filters, at a dose rate of 0.614 Gy/minute and a 56-cm focus surface distance. All mice in the treatment group were irradiated at 10 weeks old.

Table 1. Incidence of myeloid leukemia, other neoplasms and tumor-free mice, and mean survival days

Experimental groups	No. of mice[a]	Median survival time in days[b] (range)	Myeloid leukemia		Other tumor		Tumor- free mice	
			No. of case	(%)[c]	No. of case	(%)[d]	No. of case	(%)[c]
0Gy-CalR(–)	258	839 (805–865)	3	1.2	299	115.9	26	10.1[g,h]
3Gy-CalR(–)	270	697 (678–730)	60	22.2[e,f]	308	114.1	20	7.4[i,j]
0Gy-CalR(pre)	93	885 (846–924)	2	2.2	111	119.4	10	10.8
3Gy-CalR(pre)	98	722 (679–772)	16	16.3	119	121.4	7	7.1
0Gy-CalR(post)	263	896 (874–925)	0	0	213	81.0	94	35.7[g]
3Gy-CalR(post)	274	805 (768–833)	26	9.5[e]	315	115.0	48	17.5[i]
0Gy-CalR(through)	69	874 (798–898)	0	0	49	71.0	32	46.4[h]
3Gy-CalR(through)	75	731 (690–845)	6	8.0[f]	76	101.3	15	20.0[j]

[a]No. of mice refers to number of effective mice. Accidental deaths occurred due to the leakage of water bottles; 5 in 0Gy-CalR(–), 1 in 0Gy-CalR(pre), 2 in 3Gy-CalR(pre), 4 in 0Gy-CalR(post), and 8 in 3Gy-CalR(post). [b]Median survival time and the upper and lower 95% probability ranges estimated by the Kaplan-Meier method [17] (see Materials and Methods). [c]Fisher exact test for the incidence of myeloid leukemia and tumor-free mice was performed. [d]Percentages > 100% are due to multiplicity of tumor incidences. [e]3Gy-CalR(–) vs 3Gy-CalR(post) ($p<0.0001$). [f]3Gy-CalR(–) vs 3Gy-CalR(thru) ($p<0.01$). [g]0Gy-CalR(–) vs 0Gy-CalR(post) ($p<0.001$). [h]0Gy-CalR(–) vs 0Gy-CalR(thru) ($p<0.0001$). [i]3Gy-CalR(–) vs 3Gy-CalR(post) ($p<0.001$). [j]3Gy-CalR(–) vs 3Gy-CalR(thru) ($p<0.01$).

Calorie restriction procedure

Mice were subjected to four dietary conditions on the basis of the timing of CalR and thus divided into four groups: i.e., no restriction, CalR(–); preirradiation restriction (6–10 weeks old), CalR(pre); postirradiation restriction (from 10 weeks old to death), CalR(post); and a group subjected to lifetime CalR [from 6 weeks old to death, CalR(through)]. All of these groups were subdivided into two groups at 10 weeks old: those receiving 3-Gy irradiation or no irradiation (3 or 0Gy-) (see Irradiation section). Namely, there were eight groups; 3Gy-CalR(–) and 0Gy-CalR(–) groups, 3Gy-CalR(pre), and 0Gy-CalR(pre) groups, 3Gy-CalR(post) and 0Gy-CalR(post) groups, and 3Gy-CalR(through) and 0Gy-CalR (through) groups. The number of animals in each group is shown in Table 1. Identically designed cohort studies were combined; thus, animal numbers shown in Table 1 are different among the experimental groups.

Noncaloric restricted groups were fed a 95-kcal diet from 6 weeks old until death. Mice in the CalR(pre) groups were fed a 65-kcal diet from the start of the experiment, i.e., from 6 to 10 weeks old; thereafter they were fed a 95-kcal diet. Mice in the CalR(post) groups were fed a 95-kcal diet for the first 4 weeks old, i.e., from 6 to 10 weeks old, after which their body weights were controlled between 25 and 27 g with a 60- to 95-kcal dietary regimen. Caloric intake of the CalR(post) groups, however, exceeded their body weight by about 2 g, thus, it was fixed at 65 kcal from 10 to 12 weeks old until body weight decreased to 25 to 27 g. Mice in the CalR(through) groups were fed a 65-kcal diet for the first 4 weeks, i.e., from 6 to 10 weeks old, after which their body weights were controlled throughout their lifetime from 25 to 27 g with a 60–95-kcal dietary regimen. Average caloric intake from 10 weeks old calculated was 77 kcal/week, per mouse, in the CalR(post) and the CalR(through) groups.

As in our previous study, all mice were observed throughout their lifespan. All mice—except for 8% that succumbed to leukemic sudden death—exhibiting or developing anemia, or having palpable spleens, were sacrificed during the agonal period. All sacrificed mice were confirmed to have been myelogenous and had been transplantable by transplantation assay [3]. This leukemogenicity was maintained also in p53-deficient C3H/He mice as determined by fluorescein-activated cell sorting, using c-kit, Mac-1, Gr-1, B220, sIgM, Thy1.2, and CD3, among others [10]. Conventional histological examinations were routinely performed at our laboratory [11,12]. Complete necropsies were performed and organs were examined both grossly and histologically. Tissues were fixed with 4% formaldehyde in phosphate-buffered saline, embedded in paraffin, sectioned at 4-μm thickness, and routinely stained with hematoxylin and eosin. Cause of death was identified in each case. Hepatomas observed in the present study have been described elsewhere [13].

Assay of HPCs

To monitor the number of HPCs, the number of progenitor cells per spleen and that per bone marrow were evaluated by in vivo and/or in vitro colonization assay at 10 and 14 weeks (see section, Calorie Restriction Procedure). Day-12 CFU-S were assayed by

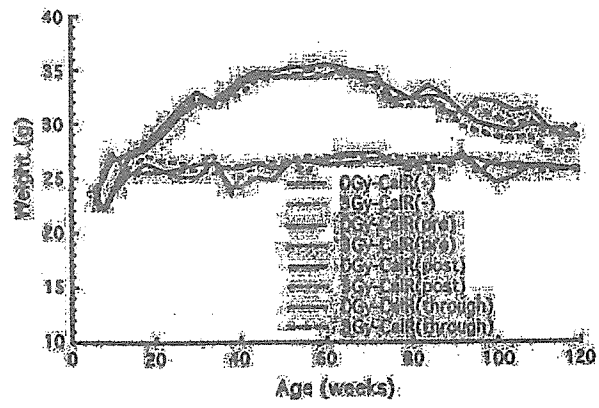

Figure 2. Changes in mean body weight vs age in weeks for all experimental groups, CalR(–), black; CalR(pre), red; CalR(post), green; and CalR(through), blue; with or without 3-Gy irradiation. Note the body weights of the 0Gy-CalR(pre) and 3Gy-CalR(pre) groups immediately returned to the non-CalR level after the dietary change at 10 weeks old, and their body weight profiles are similar to those of the controls, that is, the 0Gy-CalR(–) and 3Gy-CalR(–) groups (see text).

spleen colonization assay in accordance with the method of Till and McCulloch [14]. Mice irradiated with a lethal dose were injected intravenously with bone marrow cells or spleen cells from donor mice. Three femurs or three spleens from three donor mice of each group were pooled and assayed. Recipient mice were sacrificed on day 12 (day-12 CFU-S) after cell transfusion. Spleens with or without colonies were fixed with Bouin's solution, and surface colonies were counted.

CFU-GM were also assayed by the methylcellulose method in semisolid culture [15]. Bone marrow cells and spleen cells were cultured in alpha medium supplemented with 20% fetal bovine serum and pokeweed-mitogen–stimulated spleen-cell–conditioned medium [15]. After 7-day incubation, all CFU-GM containing more than 50 cells were enumerated.

Assay of stem cell kinetics

The bromodeoxyuridine ultraviolet (BUUV) method was used, so designated on the basis of the incorporation of bromodeoxyuridine (BrdUrd) using an osmotic minipump, followed by the specific purging of BrdUrd-incorporated cells by exposure to ultraviolet light (UV) with a peak at 365 nm (UVA), and then followed by assaying the ratio of the number of hemopoietic colonies (CFU-S, in the present study) of the purged group to that of the control group. The CFU-S–specific parameters for cell kinetics, such as doubling time, size of cell cycling (undergoing DNA synthesis) or quiescent fractions, and also size of cell-cycling fraction during a unit time interval [16] were determined. Three mice each from the 0Gy-CalR(–) and CalR groups were examined at 50 weeks old, i.e., 44 weeks after caloric restriction for the CalR groups and generally close to the time that leukemogenesis is about to become overt.

Statistical analyses

Data were stored in a computer and processed for statistical analyses using the Kaplan-Meier method for survival curves and the log-rank test [17] for statistical significance. Median survival period and the upper and lower 95% probability ranges were calculated (Table 1). Incidences of hemopoietic malignancies and tumor-free death were evaluated by Fisher's exact test (Table 1).

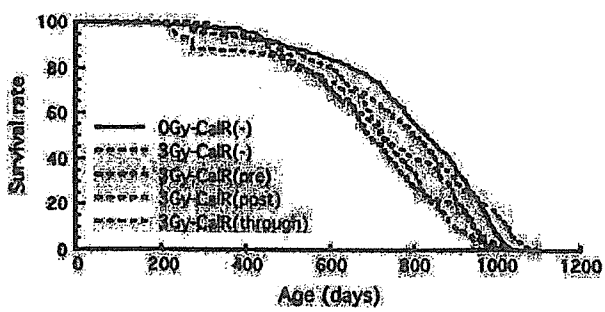

Figure 3. Survival curves for irradiated groups compared with nonirradiated control group; namely, the 3Gy-CalR(pre), 3Gy-CalR(post), and 3Gy-CalR(through) groups indicated by red, green, and blue dotted lines, respectively, are shown with those of the CalR(–) groups with or without irradiation; namely, the 3Gy-CalR(–) group indicated by a black dotted line and the 0Gy-CalR(–) group by a black solid line. For survival data for CalR groups, refer to Experimental Procedure section and Materials and Methods section. Note that the groups fed the calorie-restricted diet after 10 weeks of age without irradiation exhibit prolonged longevity. Log-rank test for mean survival curves; 3Gy-CalR(–) vs 3Gy-CalR(post) ($p < 0.0001$), 3Gy-CalR(–) vs 3Gy-CalR(through) ($p < 0.03$), 0Gy-CalR(–) vs 3Gy-CalR(–) ($p < 0.0001$).

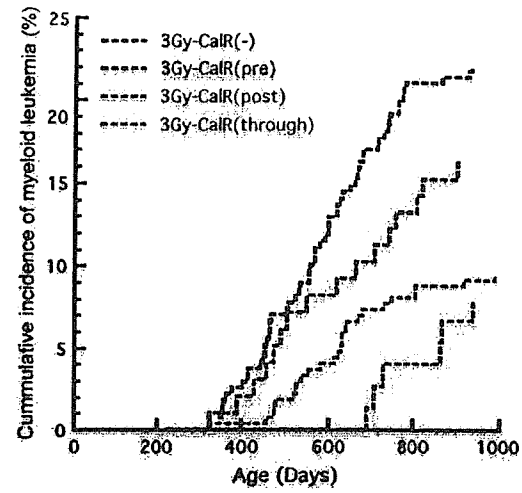

Figure 4. Cumulative incidence of myeloid leukemias. Incidences of myeloid leukemias in all the groups with caloric restriction, 3Gy-CalR(post), 3Gy-CalR(through) and 3Gy-CalR(pre) are lower than that in 3Gy-CalR(–) (see Table 1). The 3Gy-CalR(through) group shows the lowest incidence, whereas the 3Gy-CalR(post) group shows the second lowest. The 3Gy-CalR(pre) group shows a lower incidence than the 3Gy-CalR(–) group but with no statistical significance. The latency periods of the myeloid leukemias in the 3Gy-CalR(post) and 3Gy-CalR(through) groups were significantly prolonged as compared with that in the 3Gy-CalR(–) group.

Results

Effect of CalR diets on growth curves and survival

Body-weight changes in the experimental groups obtained in the present study are shown in Figure 2. There was no apparent difference in weight between unirradiated and irradiated mice in the same dietary group.

Body weights of the CalR groups given a 65-kcal diet for 6 to 10 weeks decreased to a mean weight of 22 g. Mice in these groups had lower body weights than those in the other experimental groups. Moreover, animals assigned to undergo a dietary regimen designed to maintain their weight between 25 and 27 g successfully after they reached 10 weeks old; indeed, achieved weights in this range. Changes in the body weight of the groups without caloric restriction are shown in Figure 2.

Survival curves for 3-Gy–irradiated groups with and without caloric restriction, and the 0Gy-CalR(–)group as a control are shown in Figure 3, and the comparable median survival periods (days) are listed in Table 1. Irrespective of the dietary regimen, there was a significant decrease in the lifespan of mice in all 3-Gy–irradiated groups compared with the 0-Gy groups (see, significances in legend to Fig. 3), and also in the median survival periods of mice

in the 3-Gy–irradiated groups compared with the nonirradiated 0-Gy groups (697–805 days vs 839–896 days, in Table 1). Irrespective of dietary regimen, there was a significant difference in longevity among all the irradiated groups, except for the 3Gy-CalR(pre) group, compared with that of the irradiated group without caloric restriction.

CalR prevents radiation-induced myeloid leukemias

All four irradiated groups [3Gy-CalR(–), 3Gy-CalR(pre), 3Gy-CalR(post), and 3Gy-CalR(through)] demonstrated increased incidences of myeloid leukemias as compared with the corresponding nonirradiated groups [0Gy-CalR(–), 0Gy-CalR(pre), 0Gy-CalR(post) and 0Gy-CalR(through]; 1.2% to 22.2% ; 2.2% to 16.3%; 0.0% to 9.5%; and 0.0% to 8.0%, respectively) (Table 1).

As shown in Figure 4, onset of radiation-induced myeloid leukemias was markedly delayed by CalR, specifically in the 3Gy-CalR(through) group. Total incidence of myeloid leukemias in the 3Gy-CalR(through) group was the lowest ($p < 0.01$; Fisher's exact test). The increased rate of incidence and the total incidence of radiation-induced leukemias in the 3Gy-CalR(post) group were lower than for those in the 3Gy-CalR(–) group (Fig. 4, $p < 0.0001$, Kaplan-Meier method; Table 1, 9.5% vs 22.2%; $p < 0.0001$, by Fisher's exact test). In the 3Gy-CalR(pre) group, neither onset delay, nor a significant decrease in the incidence of myeloid leukemias was observed, as compared with the 3Gy-CalR(–) group (Fig. 4, 325 days vs 321 days; Table 1; 16.3% vs 22.2%, resp.; $p = 0.217$, Fisher's exact test). However, there was no significant difference in the incidence of leukemia among the three caloric restriction groups, 3Gy-CalRs, namely, 3Gy-CalR(pre), 3Gy-CalR(post) and 3Gy-CalR (through). When the changes in the incidence of myeloid leukemias for all of the CalR groups, except that for the 3Gy-CalR(pre) group, were examined, the increase in the incidence of myeloid leukemias noted in 3Gy-CalR(–) was prevented markedly.

Because our primary aim was to examine radiation-induced myeloid leukemias and because we used strain C3H/He, a less-inducible strain for thymic lymphomas and lymphoid leukemias, hemopoietic neoplasms other than myeloid leukemias were not focused on in our examinations. Results show that there was no significant decrease in incidence by CalR in any of the irradiated groups, 3Gy-CalRs, except for the nonirradiated groups, namely, the 0Gy-CalR (post) and 0Gy-CalR(through) groups (data not shown).

Total incidence of nonhematopoietic neoplasms showed a limited decrease in only the 0Gy groups, i.e., the 0Gy-CalR(post) (81.0%) and 0Gy-CalR(through) groups (71.0%) as compared with 115.9% in the 0Gy-CalR(–) group (see Table 1 section, Other Tumors). These neoplasms include hepatomas/hepatocellular carcinomas, pulmonary tumors, tumors in the alimentary tract, genitourinary tumors, endocrine tumors, soft-tissue tumors, and dermal and skin-appendage tumors, among others.

Changes in number of hemopoietic stem/progenitor cells during or after caloric restriction

Because hemopoietic stem cells are assumed to be possible targets for radiation-induced leukemogenesis, and the number of hemopoietic stem/progenitor cells correlates proportionally to the number of CFU in vivo (CFU-S) and/or in vitro (CFU-GM), the numbers of CFU-S and CFU-GM were evaluated. A previous preliminary evaluation revealed that the number of hemopoietic stem/progenitor cells in the CalR groups decreases at the time of irradiation (10 weeks old) compared with that in the CalR(–) groups [9]. In this study, the number of HPCs at the time of irradiation (10 weeks old) and that 4 weeks after the dietary change (14 weeks old) were solely focused on and compared with those in the bone marrow and spleen (Fig. 5).

The 0Gy-CalR mice were fed a 65-kcal diet between the 6th week and 10th week. Thereafter, the 0Gy-CalR(pre) group was fed a 95-kcal diet, whereas the other 0Gy-CalR (through) group was fed the 65-kcal diet continuously. At 10 weeks old, as shown in Figure 5A (top left), the number of spleen cells in the CalR group markedly decreased as compared with that in the 0Gy-CalR(–) control group (1.32×10^8 vs 2.17×10^8 cells per spleen, respectively, second from the left vs far left). Although at 14 weeks old, in another CalR group, 0Gy-CalR(pre), the number of spleen cells originally assumed to be the same as that in the 0Gy-CalR(through) group did not decrease but rather increased as compared with the 0Gy-CalR(through) group (1.13×10^8 and 0.97×10^8 cells per spleen, respectively) due to the dietary change from a 65-kcal to a 95-kcal dietary regimen from 10 weeks old until 14 weeks old. In the 0Gy-CalR(post) group, CalR was not implemented until the 10th week; thereafter, in this particular experiment, the group was fed a 65-kcal diet until the 14th week. The number of splenic cells in the 0Gy-CalR(post) group had already significantly decreased by 14 weeks old, i.e., 4 weeks after the dietary change, as compared with the 0Gy-CalR(–) group (1.17×10^8 vs 2.00×10^8 cells per spleen).

In Figure 5B (middle, left), the numbers of progenitor cells (CFU-GM and day-12 CFU-S) per unit number of spleen cells are shown (from left to right). The number of colonies in vitro (CFU-GM) per 10^6 spleen cells for the 0Gy-CalR group markedly decreased as compared with that for the 0Gy-CalR(–) control group at 10 weeks old [lighter columns; $30.0/10^6$ cells for the 0Gy-CalR group, second from the left vs $191.7/10^6$ cells for the 0Gy-CalR(–) group, farthest left]. At 14 weeks old, the number of CFU-GM for the corresponding group, i.e., the 0Gy-CalR(through) group, showed a similar significant decrease as compared with the 0Gy-CalR(–) control group ($43.3/10^6$ cells vs $201.7/10^6$ cells). The 0Gy-CalR(pre) groups, whose number of CFU-GM was similarly assumed to be the same as that for the 0Gy-CalR(through) group, did not show any decrease as compared with the 0Gy-CalR(through) group

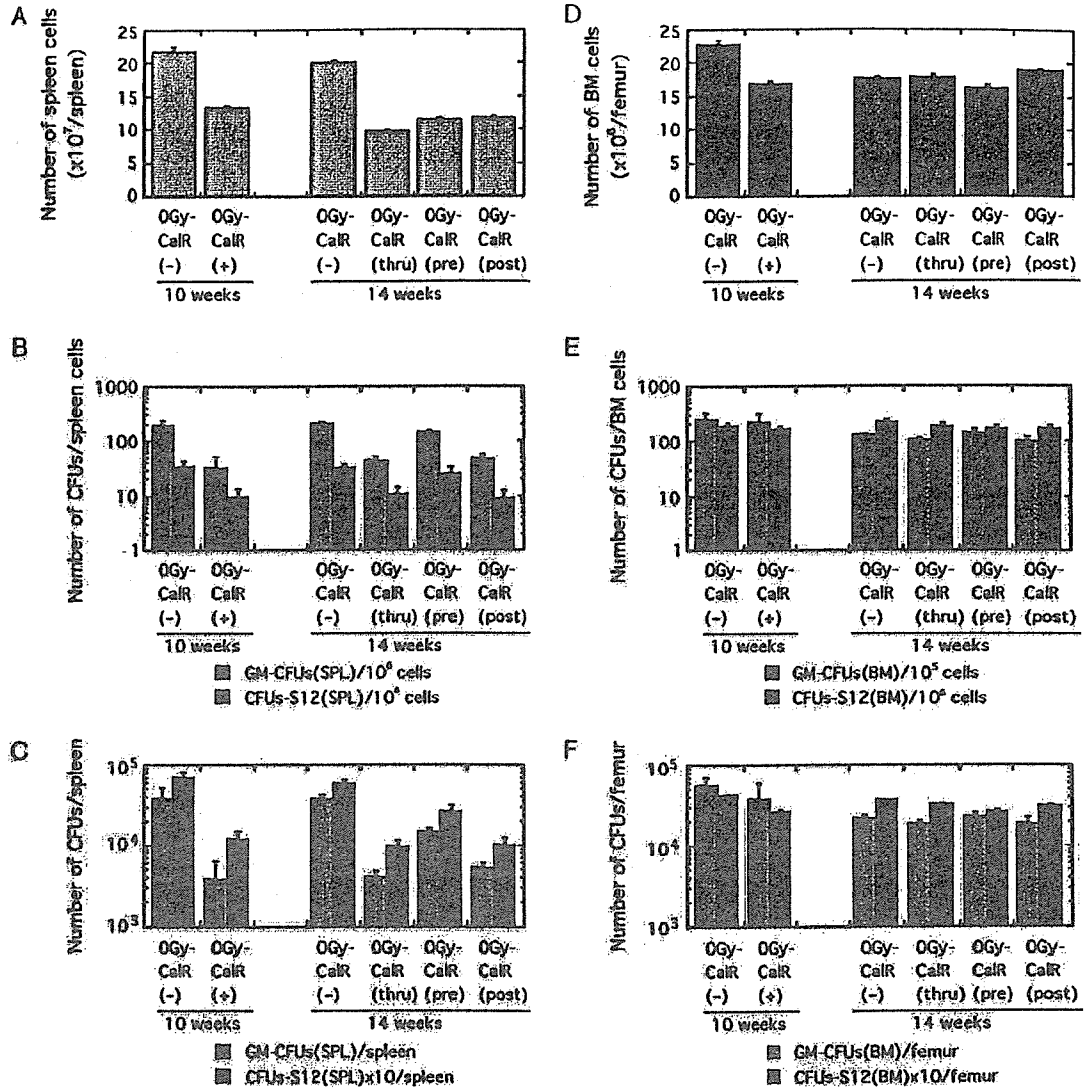

Figure 5. Number of hemopoietic cells (A,D), number of stem/progenitor cells per unit number of cells (B,E), and number of stem/progenitor cells per organ and/or tissue (C,F) are shown for the spleen (A–C) and femur (D–F). Each figure shows data at 10 weeks old, that is, 4 weeks after restriction started (left); and data at 14 weeks old, that is, 4 weeks after the dietary change (right). The right four columns represent the 0Gy-CalR(–), 0Gy-CalR(through), 0Gy-CalR(pre), and 0Gy-CalR(post) groups. For the two types of progenitor cell, the number of colony-forming units in the spleen (CFU-S) for day-12 (12D) granulocyte macrophage-colony-forming units (GM-CFU) determined by the in vitro assay was examined. Mice irradiated with a lethal dose of x-rays (810 cGy) were injected intravenously with spleen cells or femoral bone marrow cells from donor mice. For donor cells, three spleens or three femoral bone shafts from three donor mice of each group were pooled and assayed. Recipient mice were sacrificed on 12D (CFUs-S) after spleen cell transfusion. GM-CFU were assayed by methylcellulose method in semi-solid culture [15]. Spleen cells or femoral bone marrow cells were cultured in alpha medium supplemented with 20% fetal bovine serum and the pokeweed-mitogen–stimulated spleen-cell–conditioned medium (see Materials and Methods section in text). 0Gy-CalR(–) = mice fed a 95-kcal diet from 6 weeks old. CalR(thru) [CalR(through) in the text] = mice fed a 65-kcal diet from 6 weeks old. 0Gy-CalR(pre) = mice fed a 65-kcal diet from 6 to 10 weeks old, and thereafter a 95-kcal diet. 0Gy-CalR(post) = mice fed a 95-kcal diet from 6 to 10 weeks old, and thereafter a 75-kcal diet.

($136.7/10^6$ cells, $43.3/10^6$ cells, respectively) due to the dietary change from a 65-kcal to a 95-kcal dietary regimen from 10 weeks old to 14 weeks old. For the 0Gy-CalR(post) group, the number of CFU-GM significantly decreased as compared with the 0Gy-CalR(–) group ($46.7/10^6$ vs. $201.7/10^6$ cells) due to caloric restriction that started from 10 weeks old. Day 12 CFU-S (Fig. 5B, darker columns; second column of each group) also showed a trend similar to that of CFU-GM. Thus, the numbers of progenitor cells per spleen, calculated from these values, are shown in Figure 5C (bottom; CFU-GM in lighter columns and day-12 CFU-S in darker columns). When Figure 5C is

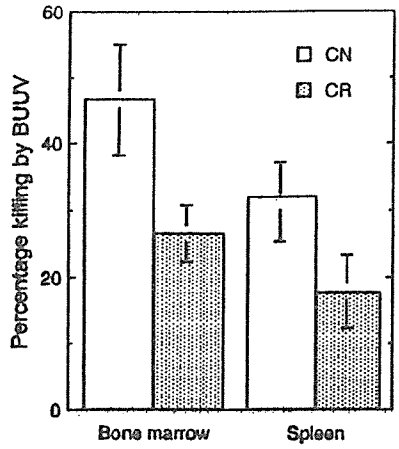

Figure 6. Percent cycling fraction (percentage killing by bromodeoxyuridine ultraviolet [BUUV] method) of hemopoietic progenitor cells, splenic colony-forming units (CFU-S) in bone marrow and spleen of mice with or without caloric restriction. BUUV assay was utilized [16], see Materials and Methods section in text. Cells from three mice each from the 0Gy-CalR(–) and CalR(through) groups were pooled and examined at 50 weeks old, that is, 44 weeks after caloric restriction. Data shown are the mean of three experiments for the spleen and of four experiments for the bone marrow. 0Gy-CalR(–) = mice fed a 95-kcal diet from 6 weeks old. 0Gy-CalR(thru) [CalR(through) in the text] = mice fed a 65-kcal diet from 6 weeks old; after 10 weeks old, the mice were fed a different diet to maintain the body weight of each mouse within 25–27 g ($p < 0.01$, the bone marrow; $p < 0.01$, the spleen).

compared with Figure 5B, all values in the figure show a similar trend but are markedly higher than those shown in Figure 5B.

The number of HPCs in each group seems to correlate with the incidence of leukemia in each group. This may be due to differences in the numbers of stem/progenitor cells between the 0Gy-CalR(–) vs 0Gy-CalR(post) groups and between the 0Gy-CalR(through) vs 0Gy-CalR(pre) groups, induced by the dietary change at 10 weeks old and its subsequent consequences. For the readers' reference, three sets of data (Fig. 5D–F) comparable to those shown in Figure 5A to C but obtained from the bone marrow are presented. None of the data for groups for the bone marrow showed any significant differences among the groups.

Changes in cell-cycling fraction of the hemopoietic stem/progenitor cells during or after caloric restriction

Effect of caloric restriction on the cell-cycle kinetics was evaluated by BUUV assay [16]. In Figure 6, the cycling fraction of hemopoietic stem/progenitor cells is represented by the percentage killing of CFU-S. In this assay, only cycling CFU-S that incorporated BrdUrd were specifically killed by UVA (365-nm peak wavelength), causing a decrease in total number of colonies assayed in the irradiated spleen. The assayed bone marrow cells, as well as spleen cells, showed a significant decrease in percentage killing in the groups subjected to caloric restriction compared with the groups not subjected to caloric restriction [46.0% in the 0Gy-CalR(–) group vs 26.0% in the 0Gy-CalR group for the bone marrow, and 31.4% in the 0Gy-CalR(–) group vs 17.7% in the 0Gy-CalR group for the spleen; at 50 weeks old]. Because the fraction that incorporated BrdUrd and was killed by UV exposure refers to that which shows a reversal of the quiescent fraction, dormant fraction; caloric restriction restored the number of stem/progenitor cells in the quiescent state, which may also contribute to the prevention of leukemogenesis.

Tumor-free death with extension of lifespan by caloric restriction

On the basis of the observation that the percentage of mice that died free of any tumor increased significantly under the regimen of caloric restriction, the following question remains to be answered. Does suppression of tumor development contribute to changes in the spectrum of diseases other than tumors, and to the extension of lifespan, or to changes in the spectrum of disease attributable to tumor-free deaths?

The percentage of mice that died free of tumors was determined by anatomic and pathological examinations at death. In the nonrestricted dietary groups, the percentage of tumor-free mice decreased from 10.1 for the 0Gy-CalR(–) group to 7.4 for the 3Gy-CalR(–) group, following 3-Gy irradiation. When caloric intake was restricted from 6 weeks until death [0Gy-CalR(through)], the percentage of tumor-free deaths increased to 46.4%, the highest, and when it was restricted from 10 weeks (0Gy-CalR(post), the percentage of tumor-free deaths was 35.7%, the second highest, among the nonirradiated diet-restricted groups [0Gy-CalR(through) and 0Gy-CalR(post); Table 1]. Following irradiation (3 Gy), the percentage of tumor-free deaths in the 3Gy-CalR(through) group was 20.0% and that in the 3Gy-CalR(post) group was 17.5%.

Although the 0Gy-CalR(through) group was expected to show the longest survival period, the median survival period and maximum lifespan in this group was limited to 874 days and 1115 days, respectively [vs 896 days and 1145 days, respectively, for the 0Gy-CalR(post) group], the reason for this is as yet unknown; presumably, CalR in the developmental stage of life may not be completely beneficial for health, but it may be beneficial for extending lifespan. Extension of lifespan was caused by changes in the spectrum of diseases attributable to tumor-free death. Cause of tumor-free death is either glomerulosclerotic renal failure, subsequent auricular thromboses with or without pulmonary edemas and increased pulmonary effusions, or cardiac failure due to progressive myocardial fibrosis and calcinosis associated or not associated with coronary sclerosis.

Discussion

Dietary restriction, particularly caloric restriction, is a major carcinogenic modifier observed during experimental carcinogenesis and significantly decreases incidence of