を割り振った。リガンド候補化合物は三次元化した後，自動ドッキングに必要な情報（水素結合情報とコンフォメーション探索時の結合回転情報）を付加した。なお，三次元化には単純なディスタンスジオメトリ法による構造立ち上げの後MMFF力場を用いた構造最適化とMOPAC-MNDO法による原子電荷計算を行なうプログラムOliveを使用し，自動ドッキングに必要な情報付加には，医薬分子設計研究所で開発したプログラム「Adam & Eve」の付属プログラムであるEve-makeを使用した。スクリーニングの結果，結合活性値と受容体－リガンド間の相互作用の強さとの間に良好な相関を得た。また，これらの化学物質のうち入手可能なものについてはCOS-1細胞を用いて転写活性値の測定も行なった。得られた情報を利用して，エストロゲン作用を有する物質が既に多く知られているフラボン類の市販化合物を対象としてバーチャルスクリーニングを実行し，21化合物を選び出した。これらの化合物の転写活性を測定したところ，11化合物に活性がみられた。

12.5　バーチャルスクリーニング

またバーチャルスクリーニングは，前述のAdam & Eveを用いてアンタゴニスト結合型ERαのリガンド結合ドメインとデータベース中の化合物の安定な複合体構造を推定することにより行なった。このプログラムは，対象データベース中のすべての化合物についてそのコンフォメーションを系統的に変えながら結合キャビティへのドッキングを試行する。その結果，タンパク質－低分子化合物間の原子同士のぶつかりや静電的な反発のない安定な結合様式とコンフォメーションを得ることができる。今回は，条件を満たす結合様式が複数得られた場合には一化合物につき最大5つの複合体構造を出力した。また，Adam & Eveのドッキング過程では低分子化合物側だけが構造最適化され受容体側はリジッドなまま扱われるため，得られたすべての複合体構造について，低分子化合物と受容体のリガンド結合キャビティを構成しているアミノ酸残基全体の構造最適化を行った後，医薬分子設計研究所で開発したタンパク質－リガンド間の結合自由エネルギーを見積もるプログラムGenBを利用して結合自由エネルギーを算出した。

12.6　考　察

内分泌かく乱化学物質のスクリーニングストラテジーにおいて，ドッキングシミュレーションによる方法は，その対象化合物の数が多いため非常に有用な手段となる。本手法によれば，従来のQSAR法に比較して，非常に高速に高精度の結果を得られ，さらに既知リガンドと構造が全く異なる化合物をも選別できるという有用性が示された。今後は，細胞系や無細胞系におけるER作用データからのフィードバック，分子内ドメイン間相互作用の解析，ポイントミューテーションの影響予測の可能性を含めたスクリーニング性能改善，及び受容体科学に向けた応用の可能性という両面への展開が期待される。

序章　化学物質と健康－低用量問題

井上　達*

1　生体と外界との相互作用

1.1　生体反応の限度幅

生体に対する化学物質の作用は，その生物がその対象物質と“遭遇”するにあたって，どれだけそれに応じた生理機能を備えているかにかかっている。自らの腸内細菌の産生するテトロドトキシンから毒性影響を受けることのない“ふぐ”自身のナトリウムチャンネルの特異な適応はこのことをよく示している。おそらく生物は悠久の昔から蓄積した体験をもとにして，外界・周囲に適応した機能を発揮しているのであるが，他方そこに備わった機能を越えた負荷に堪えることはできない。

こうした対応力の限度幅に対して許容量と呼ぶことがあるが，この呼び方はいつも正しいとは限らない。本節では一見そうした限度の範囲内に見える“possible-risk”を取り上げようとしているが，トキシコロジーはいまこうした“possible-risk”を生体が許容しているか否かの判断の難しさに直面している。ここではこの限度幅をさしあたり恕限度と呼ぶことにしよう。一般論としては生物には確かに極限の負荷に対して適応する“可塑性”も備わっており，先にみた機能的適応もその賜物に他ならない。しかしそれは長い時間軸を以って認識される次元の大きく隔たった問題であり，現時点での化学物質と生体の調和のとれた健康的な相互関係を探求する次元の問題とはいえない。

1.2　“適応反応”と傷害性

けだしトキシコロジーでは，生体影響のどこまでが適応的生体反応で，どこからが障害性変化（ここでは傷害性も同義）であるかの分界点を見定めることが課題となる。そしてその中で恕限度の占める位置も課題である。しかし截然としたその切り分けはしばしば困難なほか，それらは相互に重なり合っている面もあるので，驚くほどに適切な方法論のないことに気づく。つまりこれは新しい課題なのである。

例えば生体反応の限度内，ホメオステーシスの範囲内の変化であれば，それは生理的な変動で

＊　Tohru Inoue　国立医薬品食品衛生研究所　安全性生物試験研究センター　センター長

あり生体への障害性はないものとする見方はしばしば見受けられる。しかしながら本書で取り上げられようとしている内分泌撹乱現象などでは特に，そしておそらくもっと一般的にも，そうしたホメオステーシスの捉え方には多分に疑念が生まれつつある。この点には生物学とトキシコロジーの認識のズレもあるように見え，内分泌撹乱問題の本質もここに焦点がある。ある試験法である現象が見え[1]，他の異なった試験法でそれが見えなかった[2]，といった議論があった。果ては「科学的に」どちらが正しい，正しくないといった議論もなされた。この問題は新しい課題に該当しているので，これを混乱ないしは矛盾ととらえる人々も見られたが，本質は，多分に双方とも正しかったということに収束してゆくのではないかと考えている。

1.3 薬理と“毒理”のcontinuum[3]

化学物質と生体の相互作用，健康の保持を考えると，生体は，外界物質（の濃度）との調和のとれたバランス上に健康を維持していることが伺われる。様々の自然界の物質はもとより，紫外線や可視光のような物理的要素からはじまって，量的調節そのものは“必ずしも”自由にならないながら時間などの要素も同様の生体作用因子としてとらえられる。生体と物質の相互作用を，横軸を反応の時間軸に取った場合の種々の例を薬理学的指標と毒理学的指標を相対的に示すと図1のようになる。そこでは外界物質は，過小に過ぎれば生体の発達維持に支障を来し，過大に過ぎれば逆の面から生体障害（傷害）を引き起こす。いま生体に対する負荷からの回復という視点で考えると，“休養”のもたらす生体作用はある一面での時間軸に対する負の方向への制御ととらえることもできる。ここで人類が作り出す無機・有機の化学物質に対して生体がどのような位置関係を形成しているかについての認識も，同様の視点から理解されるわけであるが，これら

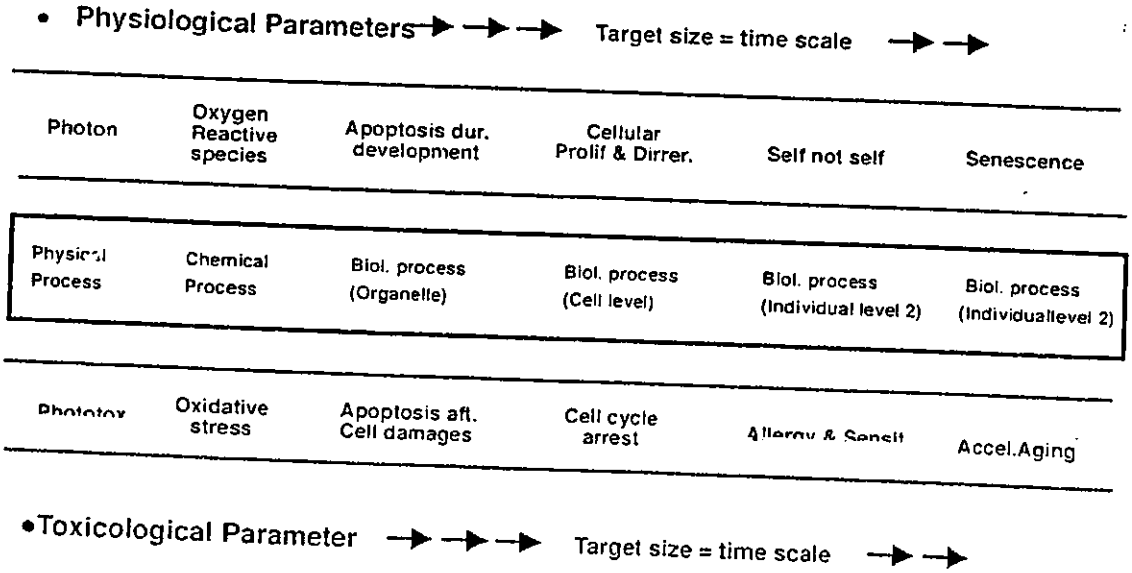


図1　生体と物質の相互作用

については同時に，生物の進化の長い歴史から見ればあまりにも経験の浅い領域に属しており，“未知”の事柄も少なくない。

1.4　恒常性の範囲内のリスク

生体には，獲得された平衡状態の維持機構が備わっていると考えられ，これはホメオステーシス（恒常性）と呼ばれるが，その背景では多次元的でネットワーク状の相互作用・拮抗関係にある様々のモメントのたえざる平衡調節が働いている。こうした関係の中では微量の物質作用は緩衝効果によってうち消されるので，これへの反応は通常の観察方法では検出されないことも知られている。観察されない認識下での事柄の生体への影響の有無や，通常観察されない事柄が生体の特殊な状態下で影響を及ぼす可能性の如何ということになると，これまで無視し得るものと判断されてきたので，当然未知の事柄が少なくない。そこでにわかに注目されているのがここに取り上げる「低用量問題[4]」である。農薬，工業用化学物質などの中に折に触れて見いだされる，ホルモン様の生体作用をもついわゆる内分泌撹乱性化学物質（環境ホルモンは俗称）は，まさにこの低用量問題を焦点としている。そこでは，野生生物の雌化現象や群集単位の縮小，ヒトでの生殖腺の異常あるいは腫瘍発生の増加などが危惧の対象として取り上げられた。結果として，それら環境中のホルモン様生体作用物質（例えば農薬，工業用化学物質）と生体（例えばホルモン受容体）との低用量レベルでの相互関係が問題の本質となっているものとの理解に至っている。これらの諸点についての参考には，米国National Research Councilの “Hormonally Active Agents in the Environment.” (1999)[5]や，WHO/IPCSがまとめたGlobal Assessment of the State-of-the-Science of Endocrine Disruptors. (2002)[6]などがあるほか，小著[7]も参照されたい。(http://www.ehp.niehs.nih.gov/who/)

2　低用量作用への認識

2.1　はじめに

毒性試験とは，障害性（ここでは傷害性も同様）限度試験であり，障害性の観察される限度を見極めることによって，その限度以下の用量における安全性を担保しようとするものである。もしこの前提が崩れるならば毒性試験による安全性の担保は，別の方法によらざるを得ないが，低用量問題は，そうした一環として登場した。このものは，①閾値の有無　②相乗性・相加性の有無，そして，③高用量からの外挿性の可否，反応の線形－非線形用量相関問題，などの諸点に分けて問題提起された。しかし実際にはこれらは相互に連関したひとつの問題である。反応性が線形用量相関を示すことが確かであれば，高用量から直線外挿性に低用量反応が想定可能であり，

低用量域に閾値があれば実質的には相乗・相加問題は発生しないからである。これらについてトキシコロジー領域に個々の具体的なデータは必ずしもなかったかも知れないが，種々の生物学的事象からくる生物学的蓋然性からみると，これらの命題の否定はもとより単純な事柄ではなかった。2000年10月，米国EPAは，ノースカロライナ州で，従来求められてきた無作用量（NOEL）や無毒性量（NOAEL）よりも低い用量域[1]で，いま内分泌撹乱問題で対象となっているようなパラメータに該当する新たな影響が観察され得るものかを問う「低用量問題に関するワークショップ」を開催した。その記録は，EPAのwebsite[4]に紹介されているのでここではふれないが，この会議以後，少しずつ低用量作用に関連する報文がでてきた。それらの諸説に収斂の気配は見えないが，双方にある方向性が認められるので，いずれそれらを整理する機会も近いものと考えられる[7~21]。

2.2 閾値の有無

閾値の有無に関する証明は実質的には生物統計学的に用量相関のモデル型から導き出すことになる。現象面からのそれには，例えば子宮肥大試験でのリガンドの用量に応じた子宮の肥大変化がロジスティックないしはシグモイド・カーブを取ることを以て知られる。因みにロジスティック・カーブの無閾値性はそれ自体では決定論とはならないが，低用量域で限りなくX軸に漸近するという意味で無閾値性を示唆している。EPAのEarl Grayは，抗アンドロジェン作用を持つ物質の種々の雌化指標が同様のロジスティック・カーブもしくはS字状曲線をとっていて，調べた限りでベースラインレベルまで接近したと述べている[22,23]。内分泌撹乱化学物質の疑義のある物質の多くがリン脂質からなる細胞膜をたやすく透過すること，従って，受容体1分子と化学物質1分子が反応するものと考えると，反応性は充分に低用量域に達することにならざるを得ないことなどがこの無閾値仮説の原点であった。事実，ホメオステーシスの環境を切り離した実験系では，*in vivo*試験でさえも極めて低い用量で様々の反応が生ずることがすでに知られている[24]。十分に低用量の領域でのリガンドの受容体との会合は当然確率的に低くはなるので，近年発がん性領域でも用いられる“practicalな”閾値はあるものと考えられる。

2.3 相乗性・相加性の有無

この問題に該当するデータとしては，かつてSoto[25]が複合アッセイ系確立の可能性を論じた報告が原点になると思われるが，この課題に真正面から取り組んだという意味で，最近注目されるのは，ロンドン大学のKortenkampのグループによる相加性に関する報告である[26~28]。彼らの一連の報告は，報告者らの文中にあるような相乗性（synergy）を意味しないが，明らかな相加性（additives）を確認したという意味でその結論は重い。先のE.Grayもvinclozolinと

procymidoneの相互作用が相加的であったとしている[29]。

2.4　反応の線形－非線形用量相関問題

この問題に関するデータは，従来のNOELやNOAELなどよりも低用量で何らかの変動パラメータが観察された，という形で間接的に示される。先のE.Greyは，vinclozolinで，このものの抗アンドロジェン作用が従来の無作用量レベル（数千mg/kg）より低いレベル（100～200mg/kg）で肛門・生殖突起間距離の短縮など様々のパラメータに雌化傾向を生ずることを報告した[22,23]。Bisphenol Aに関連したデータもこのところ数多く認められる。九州大学の粟生（Aou）らによれば，Wistar系の妊娠ラットへのBisphenol A 1.5mg/kg（NOAELは50mg/kg）を投与し，仔の成育後のオープン・フィールドテストにおける行動と，脳の青斑核（locus ceruleus）の小型化など，雌化傾向が見られたと報告している[30]。なお，こうした行動観察については，Grayらも，anti-androgenic chemicalでの結果を追加している[31]。わが国の環境省では，この低用量影響を検出する試験法の開発研究の一環として，改良一世代試験の検討を進めている[32]。その中で，di-cyclohexyl phthalateによるF1世代における8，40μg/kg/dayでのERαmRNAやARのmRNA発現の亢進（従来のNOEL/NOAELは500mg/kg/day［肝重量増加］）やdi-2-ethylhexyl phthalateによるF1世代における50μg/kg/dayでの血清FSHの上昇（従来のNOEL/NOAELは100mg/kg/dayでの肝重量増加）などを観察し，従来のNOEL/NOAEL以下のレベルで，種々のパラメータの変動の見られることを明らかにしている。環境省プロジェクトの低用量における変動パラメータの中にホルモン受容体の遺伝子発現が散見されることは，前節での考察と符合して意味がある。

3　今後の方向性

3.1　低用量問題とChildren's program

低用量問題を通覧すると，これが無視できない生物学的蓋然性を持つことが分かるが，具体的なデータの多くは胎生期間中の形態形成期や，新生児の急激な発育期に関連したものであることに気づく。このことから見ても，内分泌撹乱物質問題そのものが胎児・新生児を含む小児の問題（Children's program[33]）の重要な柱となってゆくことは疑いない。ヒトでの現存疫学データが充分な役割を果たしていない現状にあっても，今日までの結果が示す内分泌撹乱問題の本質に関わるchildren's programの生物学的蓋然性は，明らかに高いと考えられるからである。

3.2 毒性学のパラダイムシフト

低用量問題に取り組む中で，たくさんの事柄が明らかになってきた。ここで課題となった「性の可塑性」なども，多分に生命存続のための生物に備わった知恵であった。それが裏目に出た形でこの問題は発生している。その中には，様々の生命におしなべて影響の及んでいる事象が見いだされたが，他方，充分な生物学的蓋然性を持ちながらも齧歯類－霊長目－ヒトへの外挿性の明らかに否定されている事象もある。本稿で取り上げた齧歯類の性的二型核の変化などもこれに属し，霊長目での方法論は，また異なったものとなることを意味している。けだし蓋然性の蓋然性たる所以である。そうした中にあってもホメオステーシスの範囲内でのリスク，薬理と“毒理”のcontinuumに重なった生体障害の可能性は，毒性学の方法論の新たな段階を期するパラダイムシフトといえよう。低用量問題に関するノースカロライナ会議が従来の試験法そのものに疑問を投げかけることになったのは，こうした背景があってのことと考えている。

3.3 トキシコジノミクス[33]

先にホメオステーシスの陰に隠れていて表面的には“見えない”現象を見いだすことが新しい課題となることにふれた。これに役割を果たすものと期待される手法として，マイクロアレイやDNAチップによる遺伝子の大量発現技術の試行的普及が進んでいる。それらの“ゲノム発現情報とリンクして包括的に把握される”比較的大容量の分子生物学的情報は，-omicsの接尾語を付してジノミクス，プロテオミクス，メタボノミクスなどと呼称される新しい生物学領域を形成しつつある。これらのトキシコロジーへの適用がトキシコジノミクスである[34]。例えばDNA障害を伴った発がん物質の低用量域での閾値現象が，p53欠失動物では，無閾値性に観察され，通常動物でのそれは，修復に関与することが明らかになりつつある。このような通常観察されない静止変化や閾値現象は，トキシコジノミクスによって，“見えてくる”，可視化される。多次元的でネットワーク状の相互作用・拮抗関係にある様々のモメントの堪えざる平衡調節も可視化される，そういったことが期待される。これを通じていま明らかにされようとしている低用量問題は，今少し論理的な構成をもった現象として理解されるようになるものと思われる。

低用量問題は，内分泌撹乱物質問題を契機として，ヒトと外界との相互関係を探る本質的な生物学の課題の一つになろうとしている。

文　　献

1) Nagel SC *et al.*, Relative biding afficity-serum modified access (RBA-SMA)

8

assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol, *Environ Health Perspect*, 105, 70-76 (1997).

2) Cagen SZ *et al.*, *Toxicol Sci.*, 50, 36-44 (1999).

3) Inoue T., Toxicogenomics-a new paradigm of toxicology.In, T.Inoue, WD.Pennie (eds.), Toxicogenomics. Springer, Tokyo, Berlin 2003, pp.3-11.

4) 米国NTPでは，「一般的に毒性試験で用いる用量よりも低い用量」として問題提起を行った (http://nipserver.niehs.nih.gov/htdocs/liason/FinalRptLowDoseFR.html).

5) WHO/International Programme on Chemical Safety; Eds, D.Damstra, S.Barlow, A.Bergman, R.Kavlock, G.van der Kraak., Global Assessment of the State-of-the-Science of Endocrine Disruptors.World Health Organization, 2002, pp.180. (http://www.ehp.niehs.nih.gov/who/).

6) National Research Council, Hormonally Active Agents in the Environment. National Academy Press, Washinton, D.D., 1999, pp.430.

7) 井上達，五十嵐勝秀，関澤純，内分泌かく乱物質の作用機序からみたヒトへの健康障害，日医会誌，127，197-201 (2002).

8) Honma S *et al.*, Low dose effect of in utero exposre to bisphenol A and diethylstilbestrol on female mouse reproduction, *Reprod Toxicol*, 16, 117-122 (2002).

9) Palanza P *et al.*, Exposure to a low dose of biosphenol A during fetal life or in adulthood alters maternal behavior in mice, *Environ Health Perspect*, 110, Suppl.3, 415-422 (2002).

10) Schonfelder G *et al.*, In utero exposre to low doses of bisphenol A lead to long-term deleterious effects in the vagina, *Neoplasia*, 4, 98-102 (2002).

11) Schonfelder G *et al.*, Parent bisphenol A accumulation in the human maternal-fetal-placental unit, *EnvironHealth Perspect*, 110, A703-707 (2002).

12) Tinwell H *et al.*, Normal sexual development of two strains of rat exposed in utero to low doses of bisphenol A., *Toxicol Sci.*, 68, 339-348 (2002).

13) Wetherill YB *et al.*, The xenoestrogen bisphenol A induces innapriopriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells, *Mol Cancer Ther*, 1, 515-524 (2002).

14) Ashby J., Testing for endocrine disruption post-EDSTAC: extrapolation of low dose rodent effects to humans, *Toxicol Lett*, 120, 233-242 (2001).

15) Markey CM *et al.*, In utero exposre to bisphenol A alters the development and tissue organization of the mouse mammary gland, *Bio Reprod*, 65, 1215-1223 (2001).

16) Markey CM *et al.*, The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A,*Environ Health Perspect*, 109, 55-60 (2001).

17) Gupta C., Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals, *Proc Soc Exptl Biol Med.*, 224, 61-68 (2000).

18) Elswick BA *et al.*, Effect of different sampling designs on outcome of endocrine

ANALYTICAL SCIENCES APRIL 2004, VOL. 20

The Best Paper in Bunseki Kagaku, 2002

Screening of Endocrine Disrupting Chemicals Using a Surface Plasmon Resonance Sensor

Kazunobu ASANO,† Atsushi ONO, Setsuko HASHIMOTO, Tohru INOUE, and Jun KANNO

New Business Development, Biacore K.K., Koyo Bldg. 5F, 2-9-5 Shiba Koen, Minato-ku, Tokyo 105-0011, Japan

Because concern over endocrine disrupting reactions caused by chemicals to humans and animals is growing, a rapid and reliable screening assay for endocrine disrupting chemicals is required. We have developed an *in vitro* screening assay based on a hormone receptor mechanism using a surface plasmon resonance (SPR) sensor. The interaction between an estrogen receptor α (ER) and an estrogen response element (ERE) is monitored in real time, when ER is injected over the SPR sensor chip on which a DNA fragment containing ERE is immobilized. In the presence of a chemical with estrogenic activity, the ER-ERE interaction is enhanced and the kinetic parameters are altered. We have validated the assay in terms of its specificity, dose dependency, optimal reaction conditions and reproducibility. It has been shown that the assay is very reliable as a rapid and quantitative screening method to judge the estrogenic activities of chemicals.

Introduction

Recently, concern has grown that some chemicals, such as organic chloride insecticides, plasticizers and detergents can cause endocrine disrupting effects to wild animals and humans.[1] Many of them are supposed to pose endocrine disrupting activities through direct interaction with the hormone receptors, such as estrogen receptor, thus modifying or inhibiting the physiological hormonal activities.[2] Chemical safety is evaluated by a set of the toxicological tests, such as carcinogenicity, teratogenicity, mutagenicity, reproduction tests. However, they have limitation to evaluate the chronic toxicity of chemicals. Moreover, the mechanisms of the endocrine disrupting activities are yet to be elucidated and the test methods to evaluate the effects are not well established.[3]

Several test methods have been reported to detect the endocrine disrupting activities caused by hormone receptors, *i.e.* competitive receptor binding assay, a cell growth assay using breast cancer cells expressing the estrogen receptors (MCF7),[4] cell-based reporter assay,[5] an *in vivo* rodent uterotrophic test,[6,7] and a vitellogenin assay using medaka fish (Oryzias latipes).[8] Many of these methods require a long time to obtain results. Furthermore, the endocrine disrupting activities can not always be detected when the chemicals are administered to the animals due to physiological regulations concerning the animal bodies. It is not easy to detect the hormonal effects of chemicals. The existing toxicological methods are not always the best way to detect the effects which have the feedback mechanism or the effects *via* the receptors. Therefore, a novel approach is sought for the rapid assessment of the endocrine disrupting activities of the chemicals.

The hormone receptors are the ligand dependent transcription factors. For example, estrogen receptor (ER) changes its conformation upon binding of the endogenous ligand, estrogen, and binds to the specific sequence of the DNA located upstream of the target genes and activates transcription of the genes (Fig. 1). Many chemicals with diverse structures have been reported to have estrogenic activities. Due to the variety of the structure, it is unlikely that all the chemicals act with the same mechanism. Each chemical may pose a different regulatory effect on the gene expression.[9]

Recently, a surface plasmon resonance (SPR) sensor is emerging as a novel analytical instrument.[10] The SPR sensor has features that it can monitor molecular interaction without labeling the molecules in real time. It is, therefore, suitable for high throughput screening assays. Compared to the existing technologies which monitor the binding amounts at the end of the interactions, the SPR sensor is unique to be able to detect the processes throughout the association and the dissociation of the interaction. This feature enables detailed analyses of chemical effects to receptors.

We have established a cell free screening assay focusing on the hormone receptor mechanism as a high throughput screening method for the endocrine disrupting chemicals. In order to measure the interaction of the biological molecules using the SPR sensor, one of the test molecules is immobilized

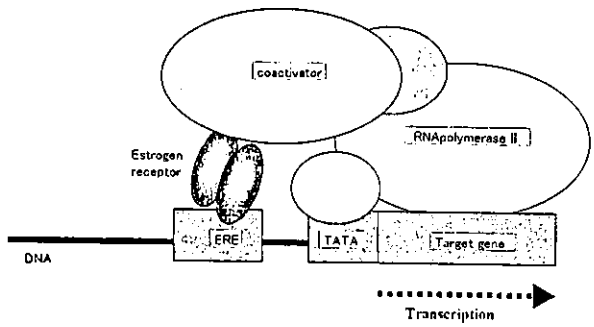


Fig. 1 Functional mechanism of the estrogen receptor in gene regulation.

† To whom correspondence should be addressed.
This is an English edition of the paper which won the Best Paper Award in Bunseki Kagaku, 2002 [*Bunseki Kagaku*, **2002**, *51(6)*, 389].

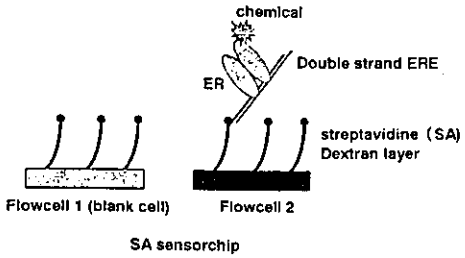


Fig. 2 Scheme of the ER-ERE assay with an SPR sensor.

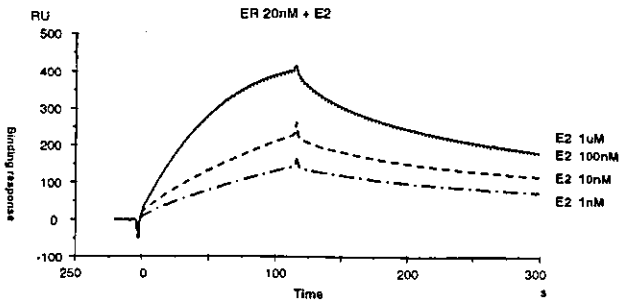


Fig. 3 Dose-dependent responses of E2.

on the sensor chip surface and a solution containing the other molecule is passed over the sensor surface at a constant flow rate through the microfluidics. A small mass changes, resulting from the binding and the dissociation of the two molecules on the sensor surface is monitored as SPR signals. The time course of the changes in the SPR signals is displayed as a curve called a sensorgram. Unlike from the conventional technologies, the SPR sensor can measure the interactions using a small amount of non-labeled samples within a short time. With regard to the interaction of the molecules, the SPR sensor can give not only the affinity of the two molecules at the equilibrium (as the dissociation constant, K_D or the affinity constant, K_A) but also the information on two molecules binding or dissociation velocity, namely the association rate constant (k_a) and the dissociation rate constant (k_d).

We have designed an assay to monitor the interaction of estrogen receptor α (ER) and the estrogen response element (ERE)[11] that is located in the promoter region of the estrogen target genes by immobilizing the DNA fragment containing the ERE sequence on the sensor chip and injecting purified ER over the sensor chip (Fig. 2). Thirty chemicals were tested for the estrogenic activities.

Experiments

Reagents and instruments

Reagent. Tricine, $CaCl_2$, $MgCl_2$, KOH, Tween 20, NaOH and HCl were purchased from Nacalai Tesque and DMSO from Sigma. Estrogen receptor (ER) was purchased from PanVera. ER was aliquoted into 5 µl and stored at –80°C. Biotinylated estrogen response element (ERE) DNA (5′-biotin-tcgagcaaagtcaggtcacagtgacctgatcaat-3′) of viterogenin gene and the anti-strand DNA have been synthesized by Nisshinbo. The synthesized oligomers were diluted with MilliQ water to 1 mg/ml and stored at –20°C. The running buffer for Biacore 3000 was prepared by filtering a solution of 25 mM Tricine, 160 mM KCl, 5 mM $MgCl_2$ (pH 7.8), 0.05% Tween 20.

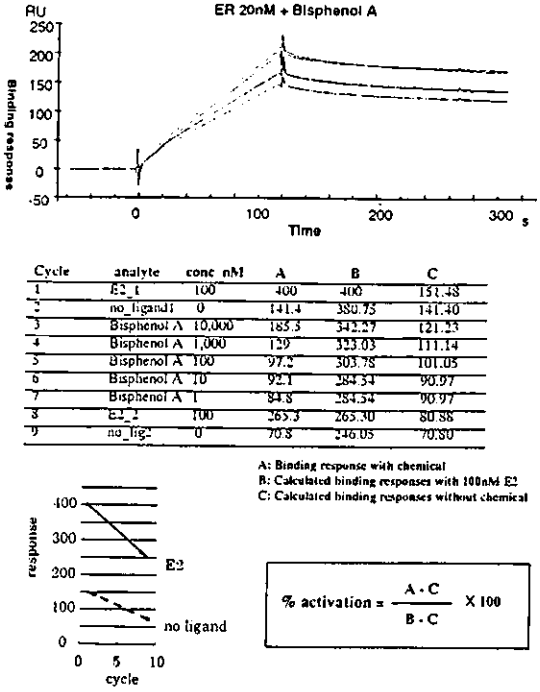
Instrument. The assay was performed using Biacore 3000 (Biacore AB), the heat block (EYELA) and the circulator (Asone). Sensor chip SA (Biacore AB) was used. Through the assay, the sample rack of Biacore instrument was cooled to 4°C by connecting the circulator to the instrument and the reaction was run at 25°C.

Operation

Immobilization of biotinylated ERE. For the immobilization of biotinylated ERE to the sensor chip, a streptoavidin preimmobilized sensor chip (Sensor chip SA) was set to the Biacore 3000 instrument and the instrument was equilibrated with running buffer. In order to stabilize the sensor surfaces, 100 mM NaOH and 50 mM HCl were injected for 30, 5 times. After checking the baseline stability, we performed immobilization of the biotinylated ERE. Biotinylated ERE (1 mg/ml) was diluted hundred thousand times with the running buffer. Then, 100 µl of the ERE solution was heated in boiling water for 5 min and chilled rapidly to denature the biotinylated ERE. The solution was injected over the sensor surface to immobilize approximately 60 RU onto the SA sensor chip surface. Then, the complementary ERE (1 mg/ml) was diluted to 100 times with the running buffer and denatured by the same method. This solution was injected for 2 min over the sensor surface where the biotinylated ERE was immobilized to form double stranded ERE on the surface. Biotin (1 µg/ml) was injected to block free SA on the sensor surface. A separate flow cell was used as a blank cell on which only biotin was immobilized.

Preparation of the test chemicals. Each chemical was dissolved with 100% DMSO to make 0.1 M stock solution, and stored at –80°C. Immediately before the assay, 1 µl of the stock solution was diluted 500 times using the chilled running buffer. Also ER stock solution was diluted to 40 nM using a chilled running buffer. A 50-µl volume of the ER solution and 50 µl of the chemical solution of each concentration were mixed to give final concentrations of 20 nM ER and 10 µM to 1 nM of the chemical. The samples were kept at 4°C in a sample rack to maintain the ER activity. 17β-Estradiol was used as a positive control. First, we prepared the various concentration of 17β-estradiol (1 µM to 1 nM) and measured the binding of ER to ERE (Fig. 3). As the binding activity of ER to ERE was plataued over 100 nM 17β-estradiol, we decided to use 100 nM 17β-estradiol as a positive control in the following experiments. We also prepared a negative control solution which did not contain any chemicals. After the preparation of samples, the samples were treated at 37°C, 5 min and rapidly cooled. The samples were then set on the sample rack for measurements.

Assay of ER and ERE. The prepared samples were injected for 2 min at a flow rate of 20 µl/min over the immobilized ERE and the blank flowcell. Injection command of "kinject" was used and the dissociation phase was monitored for 2 min. The "kinject" command is one of the injection commands specially designed for the kinetic analysis in the Biacore instrument. Upon injecting the samples using "kinject" command, the sample solution was clearly separated by two air plugs at the both ends of the sample solution from the running buffer in order to prevent the sample solution from being diluted by the running buffer. The command is also designed to monitor dissociation of the bound molecule without being disturbed by the movement of the injection needle for the set period of time. After monitoring the binding and dissociation, 100 mM NaOH and 50 mM HCl were injected 30 s each for regeneration of the sensor surfaces. All the measurements were run automatically.



Cycle	analyte	conc nM	A	B	C
1	E2_1	100	400	400	152.48
2	no_ligand1	0	141.4	380.75	141.40
3	Bisphenol A	10,000	185.3	342.27	121.23
4	Bisphenol A	1,000	129	323.03	111.14
5	Bisphenol A	100	97.2	303.78	101.05
6	Bisphenol A	10	92.1	284.54	90.97
7	Bisphenol A	1	84.8	284.54	90.97
8	E2_2	100	265.3	265.30	80.88
9	no_lig2	0	70.8	246.05	70.80

A: Binding response with chemical
B: Calculated binding responses with 100nM E2
C: Calculated binding responses without chemical

$$\% \text{ activation} = \frac{A - C}{B - C} \times 100$$

Fig. 4 Data evaluation of chemical screening.

Data evaluation

A set of the assay consisted of 5 concentrations of the test chemical, a negative control (no chemical) and a positive control (100 nM 17β-estradiol). The results were compared as the ratio to the positive control (% activation). ER was unstable and lost its binding activity to ERE during the assay period in spite of optimizing the assay conditions. We have developed an assay design to correct for any loss of the binding activity of ER over time. A positive control cycle and a negative control cycle were run at the beginning and the end of the assay. The binding responses were recorded. Based on the rate of loss in the positive and negative control samples, the binding responses of the positive and negative controls for each cycle were calculated. The enhancement of ER binding by the test chemical was expressed as the ratio to those by the positive control of 100 nM 17β-estradiol, namely as a relative activation (% activation) using the formula and the corrected binding signals, as shown in Fig. 4.

Results

Validation of the ER-ERE assay using Biacore

In order to confirm the significance of the ER assay, the binding of ER to ERE was tested with a varying concentration of ER. The binding signals increased in relation to the increasing concentrations of ER. ER did not bind to the sensor surface where no ERE was immobilized (Fig. 5). A 1 μM volume of BSA did not show any significant binding to ERE surfaces (Fig. 6). These observations indicate that the assay monitors the specific binding of ER to ERE. Comparing the results with 10, 20 and 40 nM ER, we often observed relatively low binding signals with 10 nM ER. Higher binding signals were obtained by adding a final concentration of 1 μM BSA to 10 nM ER. Due to the low protein concentration, ER was lost by absorption to the surfaces of the plastic vials and tips and the actual concentration of ER became lower than 10 nM. Based on those results, we decided to run the following assays with the final ER concentration of 20 nM.

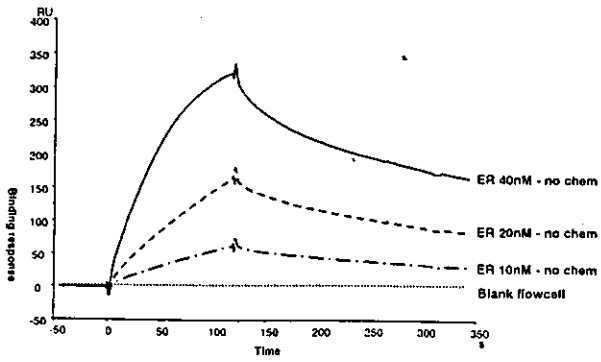


Fig. 5 Dose-dependent responses of ER-1.

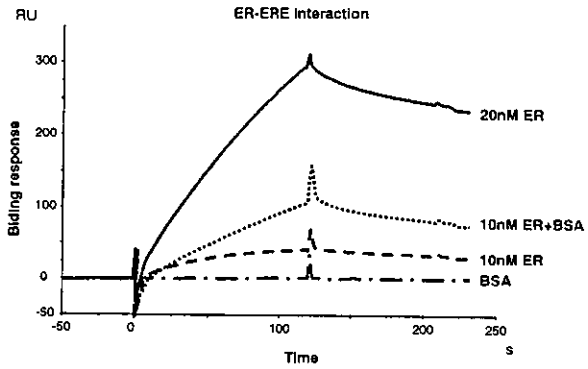


Fig. 6 Dose-dependent responses of ER-2.

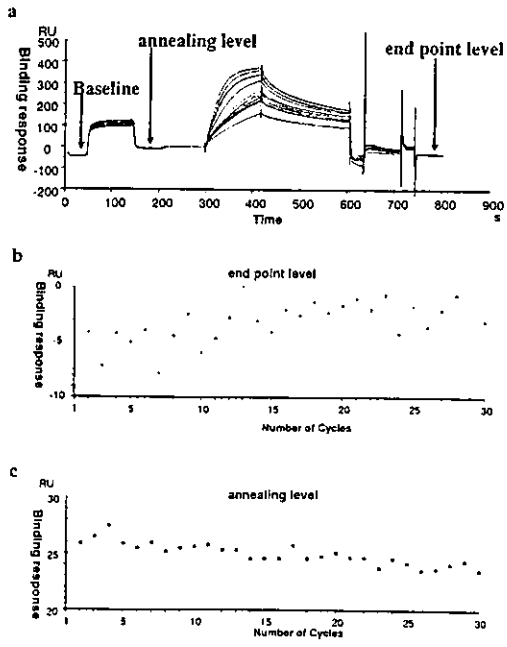


Fig. 7 Reproducibility of the ER-ERE assay.

It is important to regenerate the sensor surfaces to achieve reproducible results in the Biacore assay. We have repeated 30 cycles of the assay (Fig. 7a) and monitor the end point levels (Fig. 7b). It was confirmed that the sensor surfaces were properly regenerated and the assay showed high reproducibility.

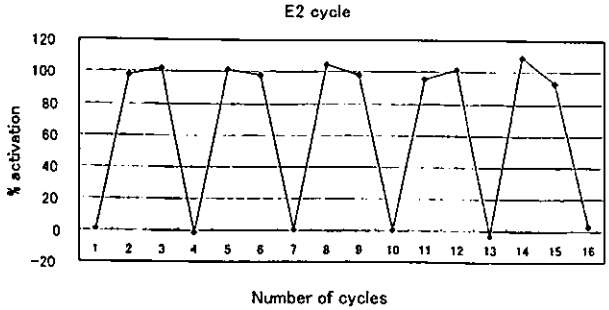


Fig. 8 Precision of the ER-ERE assay.

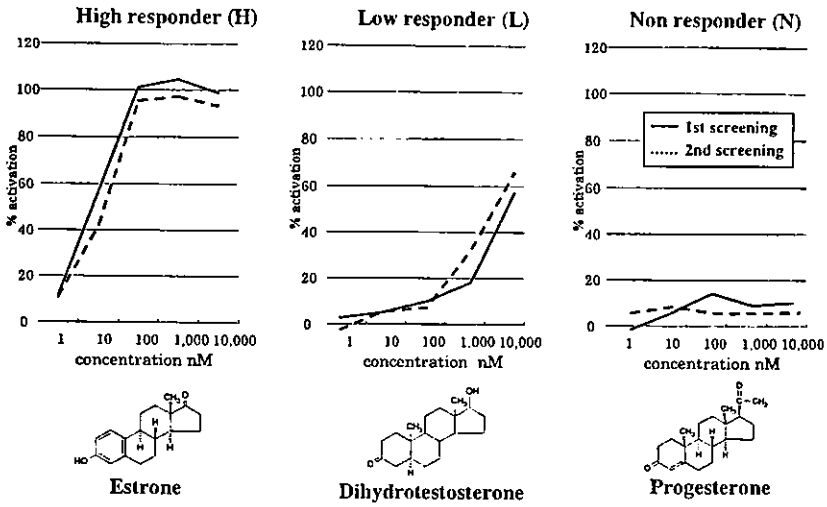


Fig. 9 Three types of chemical responses.

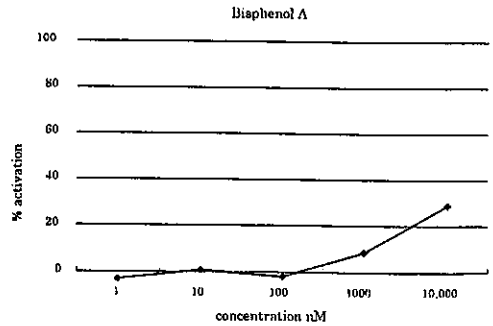


Fig. 10 Results of Bisphenol A.

The difference in the end point level was within 4 RU and the annealing level of anti-ERE was kept constant at around 25 RU throughout 30 cycles (Fig. 7c). We tested the reproducibility by repeating positive and negative controls for 16 cycles. It was shown that the results were with high precision with a CV% value of 4.68%, as shown in Fig. 8.

Screening results of 30 chemicals

We tested 30 chemicals to check the dose-dependent activation of the ER binding. One cycle of the assay took 15 min and the screening of one chemical was completed with 9 cycles in 2.5 h, including 5 different concentrations of the test chemicals, the positive and negative controls repeated twice for each control. We calculated the relative activation (% activation) using the formula shown in Fig. 4 for 30 chemicals. Based on the values of % activation at 100 nM of each chemical, chemicals could be classified into three groups (Fig. 9): the chemicals that showed more than 50% of the activation as "high responders", those with 20 - 50% as "low responders" and those less than 20% as "non-responders". The results obtained with two independent sets of screening were summarized in Table 1. 28 out of 30 chemicals showed the same results in the first and the second screening. 17β-Estradiol and its derivatives were classified to "high responders", while male hormones (progesterone) were "non-responders". Bisphenol A which is regarded as one of the endocrine disruptors, was classified among "low responders" (Fig. 10).

Furthermore, the differences in the effect of the chemicals on the ER binding activities were observed in the different shapes of the sensorgrams among those of 17β-estradiol, bisphenol A, 17α-estradiol, diethylstilbestrol (DES), tamoxifen and progesterone (Fig. 11). We have plotted the binding level at the end of the injection of ER in the presence of 1 μM of the test chemical (Y axis) *versus* the binding stability 2 min after the end of the ER injection (X axis), as shown in Fig. 12. We found that the agonists and the antagonists had significantly different patterns. The antagonists (such as tamoxifen) had a tendency to stabilize the binding of ER to ERE. The assay using Biacore indicated the possibility not only to detect the estrogenic activities of the chemicals, but to distinguish the antagonists from the agonists.

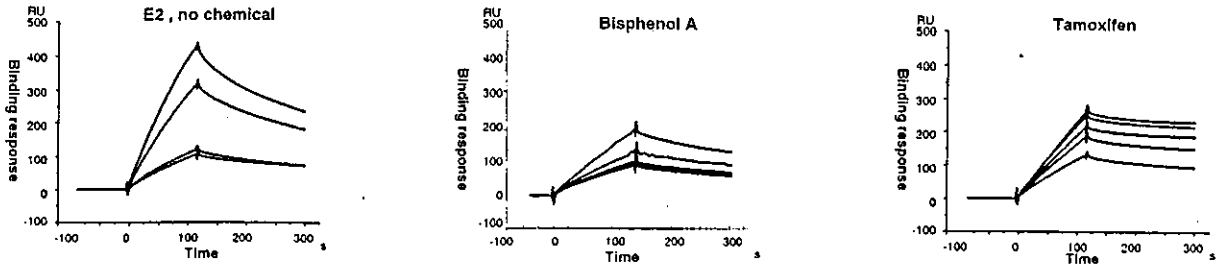


Fig. 11 Different kinetic patterns in the ER-ERE interaction.

Table 1 Results of ER-ERE screening with SPR sensor

No.	CAS No.	Name	1st Screening	2nd Screening
1	000050-28-2	Estradiol	H	H
2	000057-91-0	Estra-1,3,5(10)-triene-3,17-diol (17α)	H	H
3	000053-16-7	Estrone	H	H
4	000057-63-6	19-Nor-17-alpha-pregna-1,3,5(10)-trien-20-yne-3,17-diol	H	H
5	000362-05-0	Estra-1,3,5(10)-triene-2,3,17-beta-triol	H	H
6	000362-07-2	Estra-1,3,5(10)-triene-3,17-diol, 2-methoxy-, (17β)-	L	L
7	000068-22-4	19-Nor-17-alpha-pregn-4-en-20-yn-3-one, 17-hydroxy-	L	L
8	000063-05-8	Androst-4-ene-3,17-dione	N	N
9	000057-83-0	Progesterone	N	N
10	000501-24-6	3-Pentadecylphenol	N	N
11	005153-25-3	Benzoic acid, 4-hydroxy-, 2-ethylhexyl ester	N	L
12	001034-01-1	Gallic acid, octyl ester	N	N
13	006807-17-6	4,4'-(1,3-Dimethylbutylidene)bisphenol	L	L
14	027955-94-8	Phenol, 4,4',4"-ethylidynetri-	N	N
15	000081-92-5	Benzenemethanol, 2-[bis(4-hydroxyphenyl)methyl]-	L	L
16	000081-90-3	*o*-Toluic acid, .alpha.,.alpha.-bis(p-hydroxyphenyl)-	N	N
17	000978-86-9	4-(Triphenylmethyl)phenol	L	L
18	062625-31-4	Phenol, 4,4'-(3H-1,2-benzoxathiol-3-ylidene)bis 3-methyl-, *S,S*-dioxide, monosod	N	N
19	005384-21-4	Phenol, 4,4'-methylenebis[2,6-dimethyl-	L	L
20	005613-46-7	2,6-Xylenol, 4,4'-isopropylidenedi-	L	L
21	000084-16-2	Phenol, 4,4'-(1,2-diethylethylene)di-, meso-	H	H
22	000084-17-3	Phenol, 4,4'-(diethylideneethylene)di-	L	H
23	56-53-1	diethylstilbestrol	H	H
24	006893-02-3	Alanine, 3-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl)-, L-	N	N
25	000500-38-9	Nordihydroguaiaretic acid	N	N
26	023239-51-2	Benzyl alcohol, p-hydroxy-alpha-(1-((p-hydroxyphenethyl)amino)ethyl)-, hydrochlo	N	N
27	001050-28-8	L-Tyrosine, *N*-L-tyrosyl-	N	N
28	000145-50-6	1(4H)-Naphthalenone, 4- .alpha.-(4-hydroxy-1-naphthyl)benzylidene -	L	L
29	000446-72-0	Genistein	L	L
30	000080-05-7	Bisphenol A	L	L

H, High responder; L, low responder; N, non-responder.

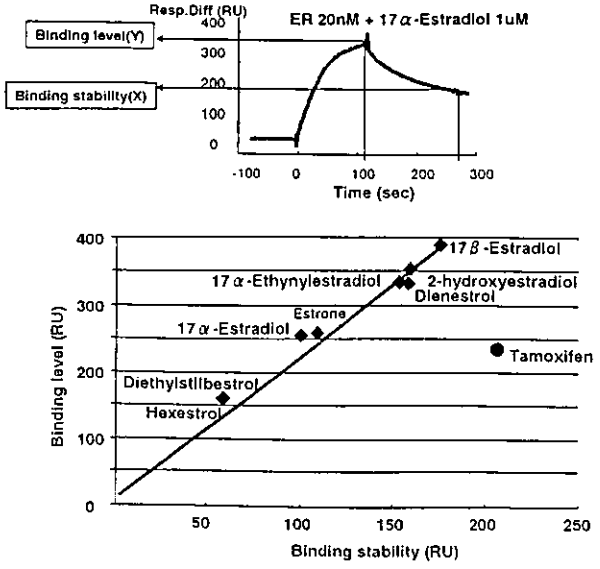


Fig. 12 Binding level *vs.* binding stability plot.

Discussion

We established a cell free screening method while focusing on the mechanism of the hormone receptor using a surface plasmon resonance sensor. We developed an assay method to detect estrogenic activities of the chemicals with changes in the binding level of ER to ERE by preincubating the chemicals with ER.

It was also suggested that the agonitst and the antagonists had different effects on the interaction of ER and ERE from an analysis of the binding level of ER during the association and dissociation processes. With the conventional end point assay used to monitor only the binding signals, it was impossible to distinguish the agonists from the antagonists. The real time analysis, which is the main feature of the surface plasmon resonance sensor enabled the classification of the agonists and the antagonists. When running the cell based hormone assay, it must be taken into account any unexpected effects of the chemicals to the other components than the receptors of the cells. On the other hand, the cell free assays simply show the

direct effects of chemicals to the receptor-signal transduction systems. Our new assay, based on the hormone receptor mechanism, can rapidly screen a large number of the chemicals for their hormonal activities.

Since other hormone receptors employ similar mechanism as ER for the activation of the gene expression, it is possible to develop same assays for other hormone receptors. A newly developed ER assay is both reliable and efficient as a primary screening method of chemicals for estrogenic activities.

Acknowledgements

This work was supported by Ministry of Health, Labour and Welfare of Japan.

References

1. "*White paper on the endocrine disrupting chemicals '99* (Japanese)", Environmental Agency, Japan, **1999**.
2. M. Nakai, Y. Tabita, D. Asai, Y. Yakabe, T. Shinmyozu, M. Noguchi, M. Takatsuki, and Y. Shimohigashi, *Biochem. Biophys. Res. Commun.*, **1999**, *254*, 311.
3. "*The interim report by the committee on the effects on health by the endocrine disrupting chemicals* (Japanese)", Ministry of Health and Welfare, Japan, **1998**.
4. A. M. Soto and C. Sonnenschein, *Biochem. Biophys. Res. Commun.*, **1984**, *122*, 1097.
5. M. Pons, D. Gagne, J. C. Nicolas, and M. Mehtali, *BioTechniques*, **1990**, *9*, 450.
6. J. R. Reel, I. V. J. C. Lamb, and B. H. Neal, *Appl. Toxicol.*, **1996**, *34*, 288.
7. OECD, OECD VALIDATION WORK ON IN-VIVO UTEROPHIC SCREENING ASSAY, **1999**.
8. "*Methods of the Biological Assays of the Endocrine Disrupting Chemicals*", ed. T. Inoue, **2000**, Springer Verlag, Tokyo.
9. P. Diel, T. Schulz, K. M. molnikar, E. Trunck, G. Ollmer, and H. Ichna, *J. Steroid Biochem. Mol. Biol.*, **2000**, *73(1 - 2)*, 1.
10. "*Real-Time Analysis of Biomolecular Interactions*", ed. K. Nagata and H. Handa, **1998**, Springer Verlag, Tokyo.
11. B. J. Cheskis, S. Karathanasis, and C. R. Lyttle, *J. Biol. Chem.*, **1997**, 272, 11384.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Toxicology 195 (2004) 19–29
TOXICOLOGY
www.elsevier.com/locate/toxicol

Exacerbation of benzene pneumotoxicity in connexin 32 knockout mice: enhanced proliferation of CYP2E1-immunoreactive alveolar epithelial cells

Byung-Il Yoon[a,b], Yoko Hirabayashi[a], Yasushi Kawasaki[a], Isao Tsuboi[a], Thomas Ott[c], Yukio Kodama[a], Jun Kanno[a], Dae-Yong Kim[b], Klaus Willecke[c], Tohru Inoue[a,*]

[a] *Division of Cellular and Molecular Toxicology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagayaku, Tokyo 158-8501, Japan*
[b] *Department of Veterinary Pathology, College of Veterinary Medicine and Agricultural Biotechnology, Seoul National University, Seoul, Republic of Korea*
[c] *Institut für Genetik, Rheinische Friedrich-Wilhelms-Univesität, Bonn, Germany*

Received 3 March 2003; received in revised form 6 May 2003; accepted 25 August 2003

Abstract

The pulmonary pathogenesis triggered by benzene exposure was studied. Since the role of the connexin 32 (Cx32) gap junction protein in mouse pulmonary pathogenesis has been suggested, in the present study, we explored a possible role of Cx32 in benzene-induced pulmonary pathogenesis using the wild-type (WT) and Cx32 knockout (KO) mice. The mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for a total of 26 weeks, and then sacrificed to evaluate the pneumotoxicity or allowed to live out their life span to evaluate the reversibility of the lesions and tumor incidence. Our results clearly revealed exacerbated pneumotoxicity in the benzene-exposed Cx32 KO mice, characterized by diffuse granulomatous interstitial pneumonia, markedly increased mucin secretion of bronchial/bronchiolar and alveolar epithelial cells, and hyperplastic alveolar epithelial cells positive for CYP2E1. But the results did not indicate any enhancement of pulmonary tumorigenesis in the Cx32 KO mice though the number of animals was small.

Keywords: Benzene; Connexin 32; Cx32 knockout mice; CYP2E1; Interstitial pneumonia; Pneumotoxicity

1. Introduction

Benzene has been reported to be a carcinogen capable of producing not only hemopoietic malignancies but also various solid tumors including lung cancers in mice, chronically exposed to it by ingestion or inhalation (Snyder et al., 1988; Huff et al., 1989; Farris et al., 1993).

Benzene toxicity and benzene-induced tumor development in the lung should be taken into consideration for the risk assessment in humans, since the lung is one of the benzene target organs and inhalation is the most common route through which humans are exposed to benzene. Furthermore, a strong relationship

* Corresponding author. Tel.: +81-3-3700-1564; fax: +81-3-3700-1622.
E-mail address: tohru@nihs.go.jp (T. Inoue).

0300-483X/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2003.08.014

between benzene exposure and lung cancer development in humans has been assumed for the past decades (Aksoy, 1985, 1989). In addition, benzene metabolites such as benzene oxide, benzene dihydrodiol and diolepoxide have been shown to induce lung tumorigenesis in mouse neonates (Busby et al., 1990). However, little information is available on the pulmonary pathogenesis triggered by benzene exposure.

Intercellular communication through gap junction proteins (GJICs) plays an important role in cellular homeostasis by regulating cell growth, cell differentiation, and apoptosis (Yamasaki, 1996). Based on this concept, alteration in GJICs has been demonstrated to be closely associated with the pathogenesis and carcinogenesis induced by chemicals, particularly by nongenotoxic agents (Yamasaki et al., 1995; Kolaja et al., 2000). Furthermore, down modulation of GJICs is known to induce cytochrome P450s by other chemicals that may be involved in benzene metabolism (Neveu et al., 1994; Snyder and Hedli, 1996; Shoda et al., 2000). We, therefore, hypothesized that GJICs may contribute also to the processes of benzene-induced pneumotoxicity and lung carcinogenesis.

As the presence and the functional role of connexin 32 (C32) gap junction protein in the mouse lung tissue have been suggested in previous in vitro and in vivo studies (Albright et al., 1990; Lee et al., 1997; Ruch et al., 1998; Abraham et al., 1999, 2001), in the present study, we explored a possible role of Cx32 in the lung pathogenesis induced by chronic exposure to benzene, using Cx32 knockout (KO) mice. For this purpose, wild-type (WT) and Cx32 KO mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for 26 weeks. Then the pathological changes were determined based on the results of histopathology, histochemistry for detecting mucin secretion, and immunohistochemistry for detecting CYP2E1 and proliferating cell nuclear antigen (PCNA). The tumor incidence in the pulmonary tissue was also compared between the benzene-exposed WT and Cx32 KO mice.

2. Materials and methods

2.1. Animals

Cx32 KO mice, from the Institut für Genetik, Universität, Bonn, Germany (Moennikes et al., 2000), were maintained as heterozygous KO mice at the animal facility of National Institute of Health Sciences (NIHS), Japan. Because the *Cx32* gene is linked to the X-chromosome, we generated Cx32 WT ($Cx32^{+/Y}$) and KO male ($Cx32^{-/Y}$) mice for this study by cross breeding female $Cx32^{+/-}$ heterozygous mice and male C57BL/6 wild type mice. The Cx32 genotypes of the neonates were identified by the standard PCR assay (Moennikes et al., 2000). The WT and Cx32 KO mice aged 8–9 weeks were used in the study. During the study, the mice were housed within stainless-steel wire cages in inhalation chambers that were maintained on a 12-h light-dark cycle. The basal pellet diet (CRF-1; Funabashi Farm, Tokyo, Japan) was provided ad libitum, except during the 6-h daily inhalation of benzene when the food was withdrawn. Water was automatically supplied throughout the study.

2.2. Benzene exposure

Benzene was purchased from Wako Chemical Company (Osaka, Japan). The mice were randomized and exposed to benzene in 1.3 m^3 inhalation chambers, as described elsewhere (Yoon et al., 2001). Briefly, the benzene vapor was generated by heating liquid benzene to 16 °C and directed into the inhalation chambers (Sibata Scientific Technology Ltd., Tokyo, Japan) with a room temperature of 24 ± 1 °C. The flow rate of benzene was about 650 l/min, and the benzene concentration in the chambers was measured at 30-min intervals during the daily exposures using a gas chromatograph (Shimadzu Co., Kyoto, Japan). The temperature and humidity in the chambers were automatically controlled at 24 ± 1 °C and 55 ± 10%, respectively. As described in the previous Section 2.1, mice were supplied water ad libitum but withdrew the food pellets during the exposure.

The WT and Cx32 KO mice were, respectively, divided into the sham-exposed control group and the benzene-exposed groups; each group was composed of ten to twelve mice. The experimental group was exposed to 300 ppm benzene for 6 h per day, 5 days per week, for 26 weeks and the sham-exposed control group was maintained under the same conditions but without benzene inhalation. Five to six mice from each group were first sacrificed after the 26-week exposure to evaluate pneumotoxicity and the remaining five to seven mice from each group were allowed to

live out their lives to further evaluate their recovery from pulmonary lesions and the incidence of the pulmonary tumor.

2.3. Measurement of food consumption and body weight

Food consumption and body weight were measured every Friday throughout the 26-week benzene exposure.

2.4. Autopsy, organ weight measurement and histopathology

After the 26-week benzene exposure, five to six mice from each group were sacrificed under ethyl ether anesthesia for autopsy. Gross morphological examination of the mice was performed and the major visceral organs were weighed and analyzed. For the histopathological examination, tissues from both lungs were fixed in 10% neutral buffered formaldehyde for 24 h. Pulmonary tissues were sliced and immediately immersed in the fixative. After routine processing, the paraffin-embedded sections were stained with hematoxylin and eosin and then examined histopathologically under a light microscope.

2.5. Immunohistochemistry and histochemistry

The avidin–biotin–peroxidase complex (ABC) method was used for immunohistochemistry to detect the expression of the P450 CYP2E1 enzyme and PCNA. After the lung tissue sections mounted on poly-L-lysine-coated slides were deparaffinized and hydrated, endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 15 min. The lung tissue sections in a Caplin jar containing 1 mM citric acid (pH 6.0) were microwaved for 10 min for retrieval of PCNA. After washing in phosphate-buffered saline (PBS, pH 7.4) for 15 min, the tissue sections were incubated with 10% normal serum at room temperature for 60 min to block nonspecific binding sites. The sections were then incubated with a mouse anti-PCNA monoclonal antibody (1:300, Sigma–Aldrich, Amherst, NY, USA) for 50 min at room temperature and a goat anti-rat CYP2E1 polyclonal antibody (1:1000, Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) overnight at 4 °C. The tissue sections were washed three times in PBS, incubated with the corresponding biotinylated secondary antibodies for 40 min at room temperature, and subsequently incubated with the ABC reagent for 30 min at room temperature. As a chromogen, 0.5% 3,3′-diaminobenzidine tetrahydrochloride was used, and the sections were counterstained with methylene blue. As a positive control for PCNA and CYP2E1, normal testis and kidney sections were used, respectively, and as a negative control, PBS instead of the primary antibodies was applied to the sections.

Periodic acid–Schiff (PAS) reaction was performed to detect mucus secretion. After deparaffinization, the tissue sections were immersed in 0.5% periodic acid solution. After washing with distilled water, the sections were incubated with the Schiff reagent for 15 min, washed with warm tap water for 10 min, and then counterstained with hematoxylin.

2.6. Statistical analysis

ANOVA was performed to evaluate the significant differences in food consumption and body weight between the nonexposed sham exposed control and benzene-exposed groups of WT and Cx32 KO mice as well as between WT and Cx32 KO mice of each group.

3. Results

3.1. Changes in body weight during the 26-week benzene exposure

No significant difference was observed between WT and Cx32 KO mice of the nonexposed sham-control group throughout the study, even when the mean body weight of Cx32 KO mice was slightly less than that of WT mice at the late stage of this study (Fig. 1). Benzene exposure induced a significant decrease in the body weight of the benzene-exposed group of both WT and Cx32 KO mice compared with the nonexposed sham-control mice. The reduction was much more marked in Cx32 KO mice (Fig. 1), which was observed after seven weeks of exposure ($P < 0.05$). On the other hand, in WT mice, a significant difference in body weight was observed after the fourteenth week of exposure (Fig. 1). Furthermore, after the twelfth week

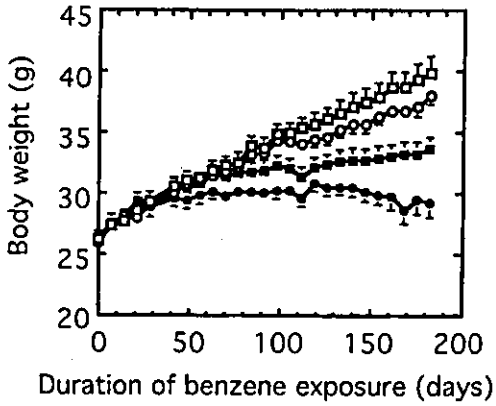


Fig. 1. Changes in body weights of WT and Cx32 KO mice during benzene exposure. Benzene (300 ppm) was inhaled for 6 h per day, 5 days per week for 26 weeks. Eleven to 12 mice per group were used. (□) WT-sham group; (○) Cx32 KO-sham group; (■) WT-benzene-exposed group; (●) Cx32KO-benzene-exposed group. There is significant difference between benzene-exposed group from the corresponding sham-control group after 10 weeks exposure for the Cx32 KO and 14 weeks exposure for WT. Vertical bars mean standard errors.

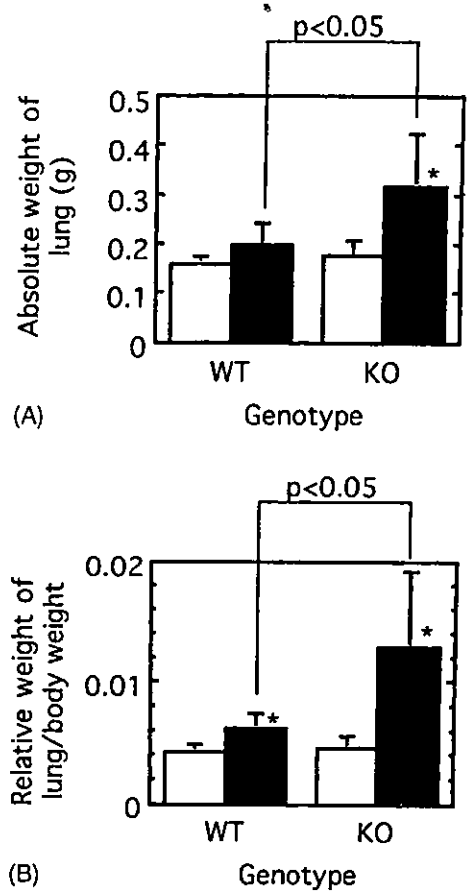


Fig. 2. Changes in weights of the lungs of mice exposed to 300 ppm benzene for 26 weeks. Open column; sham-control group, closed column; benzene-exposed group. Vertical bars mean standard deviations. Symbol (*) indicates significantly different from the corresponding sham-control group at $P < 0.05$.

of exposure, the mean body weight was significantly different between benzene-exposed WT and Cx32 KO mice ($P < 0.05$) (Fig. 1).

During the benzene exposure for 26 weeks, there had been no significant difference in food consumption between the nonexposed group and the benzene-exposed group of both WT and Cx32 KO mice and between WT and Cx32 KO mice of both groups (data not shown).

3.2. Weight of the lung

Significant increases were noted in the absolute lung weight of Cx32 KO mice and in the relative lung weights of both the WT ($P < 0.05$) and Cx32 KO mice ($P < 0.05$) after the twenty-sixth week of exposure to 300 ppm benzene (Fig. 2).

3.3. Histopathology and histochemistry

Severe diffuse interstitial pneumonia was observed in the lungs of the benzene-exposed Cx32 KO mice, which was comparable with that in the lungs of the WT mice showing much milder pulmonary lesions (Table 1, Fig. 3B and E). The alveolar walls were thickened by heavy infiltration of macrophages, the presence of a small number of lymphocytes and neutrophils, and a considerably increased number of type II alveolar epithelial cells (Fig. 3C). The proliferation of basophilic epithelial cells in the terminal bronchioles and alveolar ducts was frequently noted in the lungs of benzene-exposed Cx32 KO mice (Fig. 3F), while the lungs of benzene-exposed WT mice had mild and a few basophilic proliferating epithelial cell-proliferating foci. The numbers of mucus-secreting epithelial cells increased in the bronchi and bronchioli of both WT and Cx32 KO mice exposed to benzene for 26 weeks (Fig. 4C and D). In particular, in the benzene-exposed Cx32 KO mouse lungs, aggregates composed of mucin-secreting alveolar epithelial cells were occasionally detected (Fig. 4D).

Table 1
Pathological findings in the lungs of the wild-type (WT) and Cx32 knockout (KO) mice exposed to 300 ppm benzene for 26 weeks

Group (with or without benzene treatment)	Genotype			
	WT		Cx32 KO	
	Sham-exposed	300 ppm	Sham-exposed	300 ppm
Histopathology/no. of animals examined	6	5	5	5
Interstitial pneumonia granulomatous, diffuse	0 (0.0)	4 (80.0)	0 (0.0)	5 (100.0)
Moderate		4 (80.0)		1 (20.0)
Severe		0 (0.0)		4 (80.0)
Hyperplastic basophilic cell foci	0 (0.0)	1 (20.0)	0 (0.0)	4 (80.0)
Alveolar and bronchiolar epithelial cells		1 (20.0)		4 (80.0)
Mucin-secreting cells	0 (0.0)	5 (100.0)	1 (20.0)	5 (100.0)
Bronchial/bronchiolar epithelial cells		5 (100.0)	1 (20.0)	5 (100.0)
Alveolar epithelial cells		0 (0.0)	0 (0.0)	3 (60.0)

Number in parentheses represents the percentage (%) of the lesions.

3.4. Immunohistochemistry for PCNA and CYP2E1

The labeling indices for PCNA, compared with those of the corresponding control groups, significantly increased in both benzene-exposed WT and Cx32 KO mice; from 79.9 to 162.3‰ ($P < 0.005$) and 92.7 to 533.0‰ ($P < 0.002$), respectively (Fig. 5).

A few bronchial and bronchiolar epithelial cells of sham-control WT and Cx32 KO mice were positive for the CYP2E1 enzyme (Fig. 6A). The numbers of

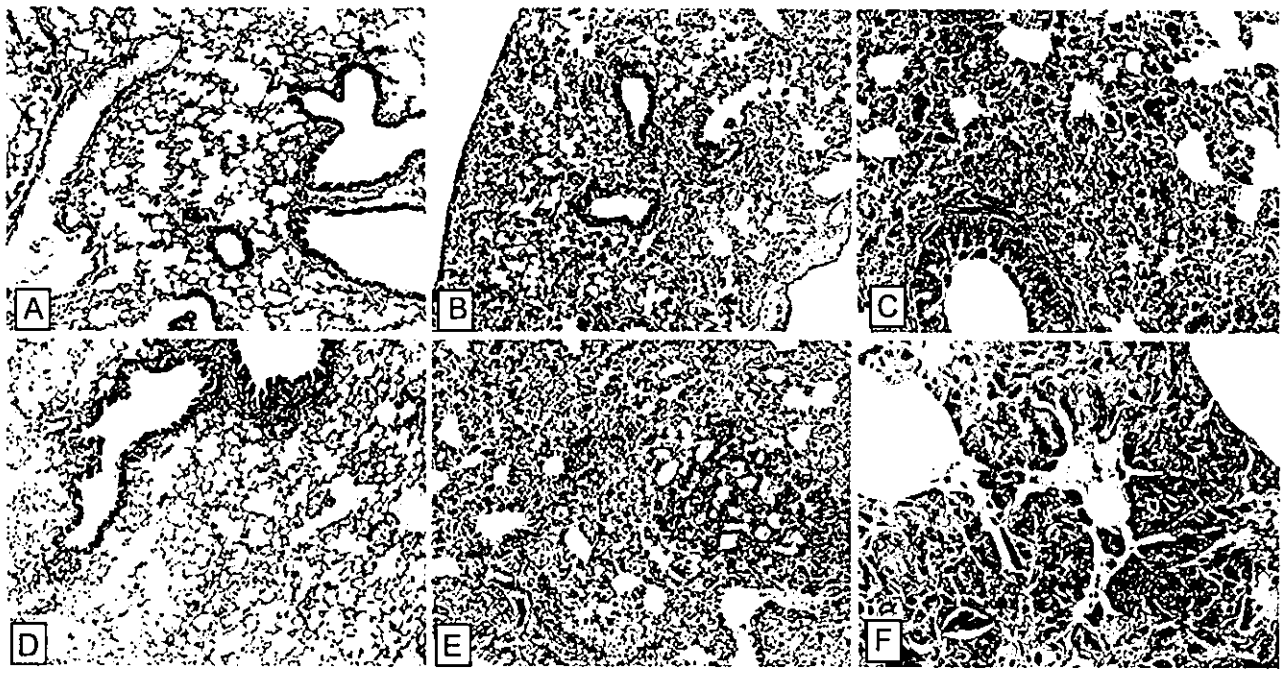


Fig. 3. Histopathological changes of the lungs of benzene-exposed WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) sham-control WT mice, (B and C) benzene-exposed WT mice, (D) sham-control Cx32 KO mice, (E and F) benzene-exposed Cx32 KO mice. Note the granulomatous interstitial pneumonia in the lungs of benzene-exposed WT and Cx32 KO mice, and basophilic epithelial cell-proliferating foci frequently observed in the lungs of benzene-exposed Cx32 KO mice (F). Original magnification: (A) ×100; (B) ×100; (C) ×200; (D) ×100; (E) ×100; (F) ×400. Hematoxylin- and eosin-stained.

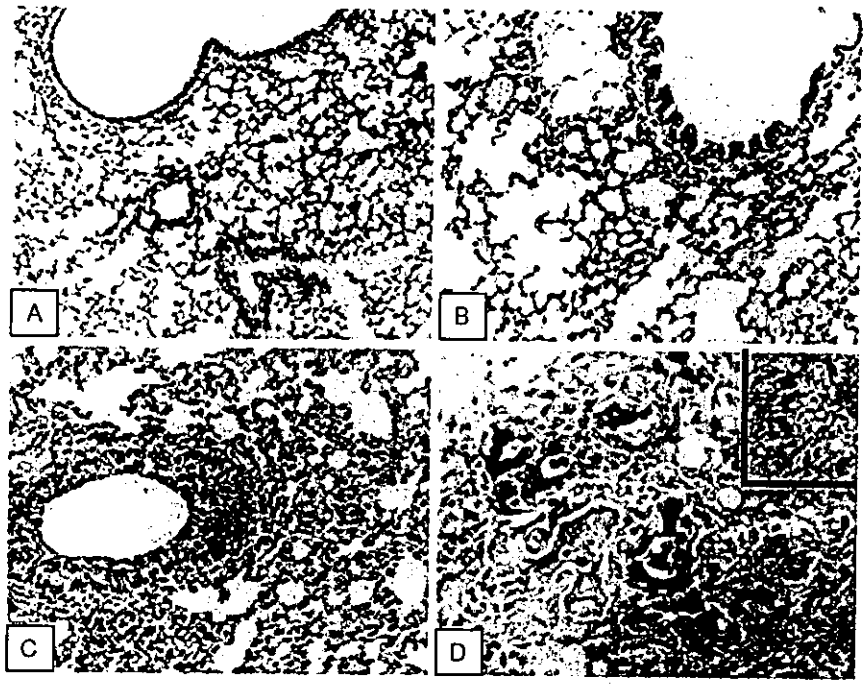


Fig. 4. Histochemistry for detection mucin secretion. (A) Sham-control WT mice, (B) sham-control Cx32 KO mice, (C) benzene-exposed WT mice, (D) benzene-exposed Cx32 KO mice. Note the enhanced mucin secretion from bronchiolar epithelial cells of WT mice (C) and Cx32 KO mice (Inset in D), and the aggregated cells releasing mucin occasionally observed in the benzene-exposed Cx32 KO mice (D). Original magnification: (A) ×200; (B) ×200; (C) ×200; (D) ×400.

CYP2E1-positive bronchial and bronchiolar epithelial cells considerably increased following long-term benzene exposure in both WT and Cx32 KO mice (Fig. 6B). The proliferating basophilic alveolar epithelial cells frequently observed in the benzene-exposed Cx32 KO mice were strongly positive for CYP2E1 (Fig. 6D), which was significantly comparable with the WT mice in which these findings were rarely observed.

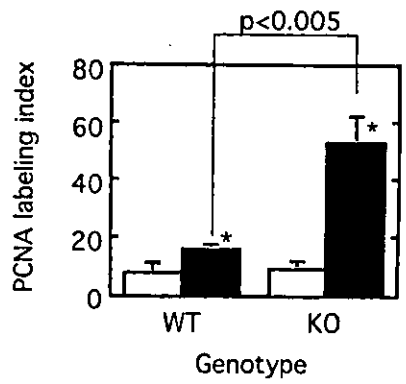


Fig. 5. PCNA labeling indices in the mouse lung tissues exposed to 300 ppm benzene for 26 weeks. Values represent the number of PCNA-positive cell per 1000 cells. More than 3,000 alveolar epithelial cells were counted under a light microscope at a high magnification (×400).

3.5. Survival curves for life time observation

Five to seven mice were randomly selected and allowed to live their life span to evaluate their recovery from pulmonary lesions and the incidence of pulmonary tumor. Survival curves for each group are shown in Fig. 7. In each group the number of mice were limited to about five to seven mice per group. There was no intermittent death during the exposure time up to 182 days (26 weeks). The sham-exposed control group indicated by open symbols, circles for WT mice and squares for Cx32 KO mice, show a longer life span than the benzene-exposed group indicated by closed symbols, circles for WT mice and squares for Cx32 KO mice. Interestingly, in the exposed group, Cx32 KO mice showed a longer life span than the wild-type mice, although the sham-exposed group does not show much difference between wild-type mice and Cx32 KO mice. During the observation period, all the mice that