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TITLE: Endogenous Retroviruses and Breast Carcinoma Development

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Human endogenous retroviruses (HERVs) have been implicated in the etiology of neoplasia including breast cancer. Like other transposable elements, their intracellular transposition may play an important role in DNA mutagenesis and carcinogenesis. To gain insight into the role of HERV in carcinogenesis, we have examined the mechanism of expression of a HERV family, HERV-K, in T47D breast tumor cell line as active expression is a prerequisite step in transposition-mediated mutagenesis by HERV. We show that HERV-K expression is enhanced by steroid hormones and that an unique set of HERV-K elements containing progesterone response element (PRE) is selectively activated by the hormones. We further showed that the PRE in the viral promoter is an effective enhancer element and the ER and progesterone receptor (PR) in T47D cells are effective transcription activators. The results suggest that these parameters are responsible for the hormone-responsive HERV-K expression. Prognosis of breast cancers is associated with their ability to respond to hormone treatment. Thus, hormone-specific HERV-K expression may be a useful prognostic marker for the progression of tumors to a hormone-resistant aggressive state.
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ENDOGENOUS RETROVIRUSES AND BREAST CARCINOMA DEVELOPMENT

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INTRODUCTION

Endogenous retroviruses have been reported in many animal tumors including human neoplasia and thus implicated in the etiology of neoplasia (1-5). The murine intracistemal A-particle (IAP) proviral sequence, reiterated 2000 times and dispersed throughout the genome, is of particular interest because of recent indication that its expression is associated with breast cancer. IAPs are rarely found in normal adult cells, but they are expressed in many different types of tumors and leukemias (4-6). In murine mammary carcinomas, the IAP gene is expressed in hyperplastic alveolar nodules induced by hormonal, chemical, or viral stimuli and in primary adenocarcinomas arising from the nodules (4,6).

The most intriguing features of the provirus-like components is their potential to act as insertional mutagens of DNA via integration of actively synthesizing extrachromosomal viral DNA to new sites in the host genome. Such transpositions induce aberrant expression of target genes such as oncogenes and genes encoding growth factors, growth-factor receptors, cytokines and cytokine receptors. Some of these alterations result in augmented growth autonomy of the host cell, contributing to neoplastic transformation (4,7-11).

Human endogenous retroviruses (HERV) have also been implicated in the etiology of neoplastic diseases. They have been found in milk samples from women with a family history of breast carcinomas (12), in breast tumors and breast carcinoma cell lines (13-16), in monocytes/macrophages residing within breast carcinomas (17), in teratocarcinoma cell lines (18-20), in leukemic cells (21), and in lung squamous cell carcinomas (22). One of the HERV family, HERV-K, is most pertinent to this report because of its elevated expression in human breast tumors. HERV-K has been cloned and shown to contain a tRNA (lysine) binding site, thus referred to as HERV-K, for reverse transcription during the first step of viral genome replication. HERV-K elements also contain a pol region closely related to type A (IAP of rodent), type D and type B (mouse mammary tumor virus) retroviruses (23). In humans, approximately 100 copies of the HERV-K sequence and more than two thousand copies of its long-terminal repeat (LTR) are scattered throughout the genome (24,25). Expression of HERV-K has been observed in the placenta, lung and kidney, in lymphocytes and leukemic cells, in teratocarcinoma cells, and most important to this study, in breast carcinoma cell lines (13,15,18,19,21,26). In a human breast carcinoma cell line T47D, HERV-K transcripts and its envelope proteins were detected by Northern analysis and by a monoclonal antibody to HERV-K recombinant envelope proteins, respectively (13,15).

Prognosis of human breast cancers is associated with their ability to respond to hormone treatment. Those responding to estrogen analog Tamoxifen treatment have a lower risk of relapse and better overall survival rate (27,28). From the study of human breast tumors and breast cancer cell T47D sublines which differ in ER expression and responsiveness to estrogen, it was shown that the loss of responsiveness to estrogen is due to mutated er with deletion, insertion, substitution and point mutation. Some of the inserts are related to the sequences of LINE or Alu (27,29). Both sequences are known to transpose to new sites in the host genome to affect expression of neighboring genes (30,31). In the present study, we have investigated the mechanism by which HERV-K expression is induced by steroid hormones to help assess the usefulness of the HERV-K expression as a prognostic marker for the progression of breast tumors to a hormone-unresponsive, aggressive state. We show that in T47D cells, HERV-K expression is enhanced by steroid hormone treatment, estrogen followed by progesterone, and that the hormones activate an unique set of HERV-K elements having the progesterone response element (PRE) in the viral promoter. We also show that the PRE in the HERV-K promoter is an effective transcription enhancer element and the ER and PR in T47 D cells are effective transcription activators.
BODY OF WORK

1. HERV-K is expressed in breast tumor cell lines.

We used RT-PCR to explore expression of HERV-K. Cells were cultured in DMEM-10% fetal bovine serum (FBS) and RNA isolated by guanidium thiocyanate procedure (32). One μg of total RNA was reverse transcribed with reverse transcriptase using oligo-dT as primer. One fiftieth of the resulting cDNA was then PCR amplified using a primer pair located within the U3 region of the HERV-K LTR to give a 291 bp product. A primer pair for the housekeeping gene elongation factor 2 (EF-2) which encodes a protein involved in elongation of peptide chain was also used to yield a 242 bp product to determine the amount of RNA used in each RT-PCR. We showed that HERV-K expression was upregulated in breast tumor cell lines T47D, BT20, and MCF-7, as compared to primary cultures of keratinocytes derived from human foreskin (Fig. 1).

Competitive PCR (33) was then employed to quantify the levels of HERV-K expression in breast tumor cell lines in comparison to other tumors (Fig. 2A). One-fiftieth of the reverse transcribed cDNA derived from 1 μg of total RNA as described above was coamplified with a dilution series of a linearized competitive template of known concentration. The competitor DNA has the same DNA sequence as the cDNA but is distinguished from the cDNA by a 50 bp deletion. Thus, the cDNA and the competitor DNA can be coamplified in the same microfuge tube with the same primer pair under identical PCR conditions, obviating variables that influence PCR amplification and allowing accurate quantification of specific mRNA species. We showed that HERV-K expression in tumor cells including breast cancer cells was at least 6 times greater than that in normal keratinocytes. The expression in two breast tumor cell lines, BT-20 and MCF-7, was as high as the HeLa epitheloid carcinoma cells and the WM164 metastatic melanoma cells. However, expression in T47D breast tumor cell line was much greater than in HeLa and WM164.

Since prognosis of breast tumors correlates with steroid hormone responsiveness, and since HERV-K expression was shown to respond to hormone treatment (13,15,27,28), we quantified its expression by competitive PCR in breast tumor cell cultures treated with or without estrogen and progesterone (Fig. 2B). The expression in the three cell lines responded differently to hormone treatment. The expression in BT-20 cells showed prominent enhancement in response to hormone treatment while enhancement in T47D cells was moderate. Expression in MCF-7 cells on the other hand did not respond to hormone treatment. Thus, the extent of expression enhancement is cell type-specific.

2. Upregulation of a selective set of HERV-K10 elements by steroid hormones.

In murine IAP family, active IAP expressions in plasmacytomas and normal lymphocytes are due to two distinct sets of endogenous proviral elements (34,35). We thus determined whether induction of expression by steroid hormones resulted from activation of a discrete set of HERV-K elements. We cloned RT-PCR products derived from RNA isolated from T47D cells cultured in the absence or presence of steroid hormones. Analysis of cDNA sequences (Fig. 3) showed that the U3 regions of cDNAs expressed in hormone treated or untreated cells are highly related in sequence, with greater than 90% homology. However, the six clones derived from hormone stimulated cells have greater homology among themselves than the six clones derived from unstimulated cells. In addition, the six clones derived from hormone stimulated cells all contained in their U3 region a TGTTCT sequence, a half site for the consensus PRE (36), while only three out of the six clones from unstimulated cells had this sequence. Two other cDNAs had TGTTAT and one cDNA had TGTTTT. Thus, steroid hormones induce expression of a selective set of HERV-K elements containing TGTTCT, suggesting that the TGTTCT sequence is an effective PRE.

3. The TGTTCT sequence in the HERV-K promoter is an effective PRE.

To determine the putative PRE activity of the TGTTCT motif, an oligonucleotide PRE(TGTTCT) containing a TGTTCT sequence and its flanking sequences (see the legend to Fig. 4 for sequence) and, as a control, PRE(TGTTAT) were synthesized and cloned upstream of the herpes simplex virus thymidine kinase (TK) promoter in pBLCAT2, a chloramphenical acetyltransferase (CAT) expression vector (37). These reporter genes, PRE/TKCAT containing TGTTCT and PRE/TKCAT containing TGTTAT, were
cotransfected with a human PR expression plasmid (pRSV-hPRB, a gift from Dr. B. O'Malley, Baylor College of Medicine) into HeLa cells treated with or without progesterone. Analysis of the resulting CAT activity revealed that the TGTTCT containing reporter gene responded well to progesterone but the TGTTAT containing reporter gene did not (Fig. 4). The results indicate that the TGTTCT motif is an effective PRE but the TGTTAT sequence is not.

4. The ER and PR in T47D cells are effective transactivators.

The fact that HERV-K expression in T47D cells responded to hormones (Fig. 2) indicated that the PR was effectively induced by estrogen acting through ERs (38,39) and was an effective enhancer. To substantiate this conclusion, we used transient transfection assays to investigate the ER and PR in T47D cells. We first examined whether these cells contain constitutively expressed PRs as previously reported (40). The PRE/TKCAT containing TGTTCT reporter gene, from here on referred to as PRE/TKCAT, was transfected into T47D cells treated with or without progesterone. The progesterone induced active expression of the PRE/TKCAT reporter gene, indicating the presence of constitutively expressed PRs (Fig. 5A). In addition, the expression of the PR gene was inducible by estrogen since in the transfected cells treated with estrogen followed by progesterone, the reporter gene expression was greater than that in cells treated with progesterone alone (cf Fig. 5B with 5A). Thus, T47D cells contain ERs which upon binding to estrogen activate expression of the PR gene to induce expression of the CAT reporter gene through the PRE. The results suggest that the transactivators ER and PR in the T47D cells and the PRE in the HERV-K promoter are responsible for the hormone-responsive HERV-K expression.

CONCLUSIONS

We showed that HERV-K expression in T47D cells was enhanced in response to hormone treatment and that an unique set of HERV-K elements containing PRE was selectively activated by the hormones. We further showed that the PRE in the viral promoter was an effective enhancer element and the ER and PR in T47D cells were effective transcription activators. Thus, the results suggest that the transactivators ER and PR in the breast tumor cells and the PRE in the HERV-K promoter are responsible for the hormone-responsive HERV-K expression.

We also showed that HERV-K expression in BT-20 cells was responsive to hormone but that in MCF-7 cells was not. Both BT-20 and MCF-7 cells were shown to contain varying ratios of wild-type and truncated ER variant mRNAs. A variant ER that has the hormone-binding domain deletion was shown to encode a dominant-positive receptor capable of transactivating an estrogen-regulated reporter gene in a hormone-independent manner. Thus differences in estrogen responsiveness of sublines of BT-20 and MCF-7 cells are related to the ratios of wild-type and truncated variant ER mRNAs (41-43). We intend to examine the sequences of the HERV-K proviral elements expressed in BT-20 and MCF-7 cells upon hormone stimulation to determine whether steroid-specific pattern of HERV-K expression, e.g., activation of PRE containing elements, is maintained or additional elements are activated since HERV-K expression in BT-20 cells responds to hormone treatment but that in MCF-7 cells does not. Examination of the structure and function of the ER and PR in BT-20 and MCF-7 cells should also reveal the effect of hormone receptor mutations on the hormone-resistant aggressive tumor phenotype and HERV-K expression, and thus the possibility of HERV-K expression as a prognostic marker in breast cancer.
REFERENCES


Fig. 1. HERV-K expression in breast tumor cell lines by RT-PCR. Cells were cultured in DMEM containing 10% FBS and total cellular RNA was isolated and treated with DNase. One μg of RNA from each cell type was reverse transcribed with reverse transcriptase (RT, +) using an oligo-dT primer. Control reactions without RT (-) were performed to test for DNA contamination in the RNA samples. One-fiftieth of the resulting cDNA was PCR amplified using primers located within the U3 region of the HERV-K LTR designed to give a 291 bp product or primers to the housekeeping gene EF-2 designed to yield a 242 bp product. The latter was used as a control for RNA loading. The absence of any detectable RT(-)-PCR product indicates that DNA contamination in the RNA sample is negligible. Thus, the intensity of the RT-PCR represents the abundance of the HERV-K mRNA in the cells. Kerat: normal keratinocytes. BT-20, MCF-7, T47D: breast tumor cell lines.
Fig. 2. Quantification of the HERV-K mRNA levels in breast tumor cells by competitive PCR. (A) Competitive PCR of breast tumor cells, BT-20 (B), MCF-7 (M), T47D (T); epitheloid carcinoma cells, HeLa (H); metastatic melanoma cells, WM164 (W); and normal keratinocytes (K) cultured in DMEM-10% FCS. (B) Competitive PCR of breast tumor cells treated with (+) or without (-) steroid hormones, estrogen plus progesterone. Cells were cultured in a phenol red free medium containing 3 volume of DMEM, one volume of L-15 and 5% charcoal stripped FBS for 24 hr, then in the same medium with or without 0.1 μM estradiol for 24 hr followed by with or without 1 μM progesterone for 24 hr. Phenol red has estrogen-like properties and FCS contains endogenous steroids which can be removed by charcoal stripping. The HERV-K expression in (A) thus reflects the stimulation by traces of steroids in the culture medium. One fiftieth of the reversed transcribed cDNA derived from one ug of total cellular RNA was coamplified with a dilution series of a linearized competitive template using the LTR primer pairs employed in the PCR depicted in Fig. 1. The competitive template has the same DNA sequence as the cDNA but is distinguished from the sample cDNA by a 50 bp deletion. Quantity of sample cDNA is determined by a plot of the ratio of the intensity of each competitor band/sample band vs. the amount of competitive DNA in the reaction.
HOWE, Chin C. 

B.

PRE

S1 GCAAGAGAGATCAAATTCTGTTACTGCTCTGTAGGAAAGAAGTACATGAGACTCCATTTTGTTCTGTACTAAGAA 80
S2 ----------------- T--- N--- ------------------- A--- G---
S3 ----------------- T--- G---
S4 ----------------- T---
S5 --------------- R--- A--- T--- C---
S6 --------------- T---

bicoid

S1 ATCACCACTCTCTAATCTCAAGTACCCAGGG-ACACAAAACTGCCGAAGGCCCCAGGGACTTCTGCCTAGGAAAGCCAG 320
S2 ----------------- T--- C---
S3 ----------------- T---
S4 ----------------------------------------------- C-------
S5 ----------------- T---
S6 ----------------- T---

RA

S1 GTATTGTCCAACCTTTCTCCCCATGTGATAGTCTGAAATATGGCCTCATGGGAAGGGAAA LOMýACCGTC~CCCAG 400
S2 ----------------------------------------------- G--- T---
S3 ----------------------------------------------- G---
S4 ----------------------------------------------- G---
S5 ----------------------------------------------- G---
S6 ----------------------------------------------- G---

myb

S1 CGACACCCGTAAAGGGTCTGTGCTGAGGAGCCATTAGTATAAGAC-G
AAGCATTCCTCTTGCAC-TTGAGACAACAGGAA 480
S2 ----------------------------------------------- N---A---
S3 ----------------------------------------------- A---
S4 ----------------------------------------------- A--- G---
S5 ----------------------------------------------- A---
S6 ----------------------------------------------- A---

AP2

S1 GCCATCTGTCCTGGCCCTCTGCTGGCAATGGTCTCTGCTATATAAAACCCGATGTGACAGTGCACCTAAGTAGTGCAGATAG 560
S2 ----------------------------------------------- CA--- T--- T--- C---
S3 ----------------------------------------------- T---
S4 ----------------------------------------------- G--- G---
S5 ----------------------------------------------- G---
S6 -----------------------------------------------

AP2

S1 GAAAGAAACGGCCTTAGGCGCTGGGACATGCGGCGAATATACCTGCTTTGTTAAAGGATTAG 640
S2 -----------------------------------------------
S3 -----------------------------------------------
S4 -----------------------------------------------
S5 -----------------------------------------------
S6 -----------------------------------------------
Fig. 3. Nucleotide sequence comparisons of the U3 regions of HERV-K cDNAs expressed in hormone-treated (A) or -untreated (B) T47D cells. (A) Cells were treated, as described in the legend to Fig. 2B, with estrogen plus progesterone. (B) cells were cultured in DMEM containing 10% FBS. Total RNA was reverse transcribed with an oligo-dT primer and a 621 bp fragment of the HERV-K LTR was PCR amplified and cloned into pCR II (Stratagene) according to the manufacturer’s instructions. Clones were randomly selected and sequenced by the DNA Sequencing Facility at The Wistar Institute. Several variant forms of PRE are seen in cDNAs (S series) expressed in hormone-untreated cells, while only the TGTTCT motif, a half site for the consensus PRE, is detected in cDNAs (T series) isolated from hormone-treated cells.
Fig. 4. Analysis of the putative PRE activity of the TGTTCT motif. HeLa cells cultured in phenol red free DMEM-5% FCS (charcoal stripped) were cotransfected with the progesterone receptor expression plasmid (pRSV-hPRB) and either the CAT reporter plasmid pBLCAT2 (TKCAT), pBLCAT2 with three copies of PRE(TGTTCT), or pBLCAT2 with three copies of PRE(TGTTAT) inserted in front of the TK promoter. pRSV-LacZ was also included in cotransfection to use as an internal control for transfection efficiency. The cotransfected cells were cultured in the presence (+) or absence (-) of 1.0 μM progesterone for 48 hr. Cell extracts were harvested and CAT activity assayed by our routine procedures (44,45). The upper panel shows the autoradiograms of products from CAT assays. The lower panel is the histograms of CAT activity in progesterone treated cells relative to TKCAT activity set at 1.
Fig. 5. Characterization of the PR in T47D cells treated with progesterone (A) or with estrogen plus progesterone (B). (A) T47D cells cultured in phenol red free DMEM-5% FCS (charcoal stripped) were transfected either with a reporter gene (PRE-TKCAT) containing three copies of PRE (TGTTC) (wt) or three copies of mutated PRE (ATAGCA) (mt). Transfected cells were treated with (+) or without (-) 1 μM progesterone for 48 hr. The CAT activity from the PRE-TKCAT reporter was elevated as compared to that from the TKCAT reporter when the cells were treated with progesterone. A reporter gene containing mutation in the PRE, PRE(mt)-TKCAT, failed to exhibit enhanced CAT activity in response to progesterone. (B) Cells were transfected and grown as in (A). Transfected cells were treated for 24 hr with 0.1 μM estradiol followed by the 24 hr progesterone treatment. Treatment with estradiol followed by progesterone resulted in greatly enhanced CAT activity in the cells transfected with the PRE-TKCAT but not with PRE (mt)-TKCAT.
LIST OF PERSONNEL

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Mei Chen, Technician