Ether à go-go Potassium Channels as Human Cervical Cancer Markers

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ABSTRACT

Ether à go-go (EAG) potassium channels display oncogenic properties. In normal tissues, EAG mRNA is almost exclusively expressed in brain, but it is expressed in several somatic cancer cell lines, including HeLa, from cervix. Antisense experiments against eag reduce cell proliferation in some cancer cell lines, and inhibition of EAG-mediated currents has been suggested to decrease cell proliferation in a melanoma cell line. Because of the potential clinical relevance of EAG, we investigated EAG mRNA expression in the following fresh samples from human uterine cervix: 5 primary cultures obtained from cancerous biopsies, 1 cancerous fresh sections in cervical cancer biopsies sections from the same patients. In addition, we looked for EAG-mediated currents in the cultures from cervical cancer cells. Here we show for the first time EAG channel activity in human tumors. Patch-clamp recordings showed typical EAG-mediated currents modulated by magnesium and displaying a pronounced Cole-Moore shift. Because EAG expression and channel activity have been suggested to be important in cell proliferation, our findings strongly support the idea of considering EAG as a tumor marker as well as a potential membrane therapeutic target for cervical cancer.

INTRODUCTION

Potassium channels play an important role in several cellular functions such as excitability, contraction, cell cycle progression and metabolism (1). In particular, some members of the ether à go-go (EAG) potassium channels family are modulated through the cell cycle (2–8) and have been suggested to be involved in tumorigenesis (9–17). Rat EAG channels expressed in frog oocytes display rectification induced by mitosis-promoting factor activation (2), and their conducting properties change during cell cycle (3). Retinoic acid down-regulates hEAG current in neuroblastoma cells (4). hEAG is transiently expressed before myoblasts fusion, which is a cell cycle-related event (5, 6). hEAG current decreases during M phase and is modulated by cytoskeletal elements (7). Channel subunits of another member of the EAG channels family, the human ether à go-go related gene (hERG), are differentially expressed throughout the cell cycle (8).

One of the most intriguing aspects of hEAG and hERG channels is their relationship to cellular transformation. Cells transfected with eag are able to grow in the absence of serum, lose contact inhibition, and induce aggressive tumors when injected into immune-depressed mice (9). EAG mRNA expression in normal tissues is mainly restricted to the brain. It is also expressed transiently in skeletal muscle and slightly expressed in placenta. On the other hand, EAG mRNA is expressed in several cancer cell lines including HeLa, MCF-7, SHSY-5Y, and IGRI from carcinoma of the cervix, breast tumor, neuroblastoma, and melanoma, respectively (9, 10). Despite the major expression of EAG in normal brain (9), endogenous EAG-mediated currents have been reported only in myoblasts (6) and in the tumoral cell lines SHSY-5Y (4), MCF-7 (11), and IGRI (10). Eag is expressed in the tumor cell line HeLa (9); however, no EAG-mediated currents have been described in these cells.

Expression of eag and EAG-mediated currents in transformed cells seem to be important events for cell proliferation, because inhibition of EAG expression with antisense oligonucleotides reduces cell proliferation in some cancer cell lines (9). Similarly, EAG-mediated currents inhibition by imipramine have been suggested to decrease cell proliferation in IGRI cells (12). Furthermore, cells expressing nonconducting EAG channels fail to induce tumor formation when injected into immune-depressed mice. EAG mRNA has been described not only in tumor cell lines but also in several human tumors including mammary gland, liver, prostate, uterine cervix, ovary, endometrium, colon, and thyroid; a significant percentage of epithelial tumors show robust Eagl expression (13), whereas hERG channels are expressed in several cancer cell lines from different histogenesis including leukemic cells (14–16) and frequently expressed in biopsies from endometrial cancer (17).

Because EAG might be considered as a tumor marker and an interesting therapeutic target for cancer, we investigated the presence of EAG mRNA in 5 primary cultures from human cervical cancer fresh biopsies, 1 cancerous cervical tissue, and 12 control noncancerous cervical fresh biopsies obtained by hysterectomy indicated because of benign gynecologic conditions. Additionally, we looked for EAG channel activity in the cancer cells from primary culture.

MATERIALS AND METHODS

Biological Samples

Cervical cancer biopsies were obtained from patients registered at the Instituto Nacional de Cancerología in Mexico City following the local ethical
considerations. We studied only biopsies from patients who had not received any anticancer therapy (chemotherapy, radiotherapy, or surgery). Tissue fragments –0.25 cm³ were obtained following an office procedure and placed in a plastic tube containing cell culture medium (described below). Obtained samples were diagnosed as Epidermoid Cancer Federation Internationale de Gynecologie et d’Obstetrique stage IIA (1), IB (1), IIIB (2) and Adenocarcinoma IIB (1). Control, normal cervical biopsies were obtained by hysterec- tomy indicated because of benign gynecologic pathology from patients regis- tered at the Hospital General “Dr. Manuel Gea González” in Mexico City also following the local ethical considerations. Tissue fragments ~1 cm³ were obtained from cervix during surgery and placed in a plastic tube containing RNA later (Ambion, Austin, TX) solution. All of the control cervical samples came from patients with negative pap smears (normal cervix).

Cell Culture

Cervical cancer biopsies were immediately placed in cell culture medium prepared with high glucose-DMEM, heat-inactivated fetal bovine serum (10%), and antibiotics and transported to the laboratory. Isolated cells were obtained by mechanical fragmentation of the tissue, distributed in Petri dishes, and incubated at 37°C (95% humidity and 5% CO₂ atmosphere). When reaching confluence at 80%, cells cultures were trypsinized up to three passages and then frozen in liquid nitrogen. Experiments were performed with cultures having from one to three passages to avoid additional cellular changes due to excessive passages.

PCR Amplifications, Southern Blot Analysis, and Sequence

Eag1. Total RNA was extracted from primary cultures of cervical cancer cells and directly from normal cervical tissue with Trizol reagent (Invitrogen, Grand Island, NY). hEAG-transfected Chinese hamster ovary (CHO) cells (kindly provided by Walter Stühmer, Max-Planck-Institut für experimentelle Medizin) were used as positive control. RNA was subjected to reverse tran- scription (RT) and then frozen in liquid nitrogen. Experiments were performed with cultures having from one to three passages to avoid additional cellular changes due to excessive passages.

Immunohistochemistry. Cervical cancer biopsies were fixed in a buffered 4% formaldehyde solution. Direct immunohistochemistry was performed as follows: samples were dehydrated in a graded series of EtOH and embedded in paraffin. Serial sections, 5 μm, were cut on a rotation microtome, mounted on glass slides, and deparaffinized using xylene and a decreasing series of EtOH. After washing with PBS, slides were immersed in citrate buffer (0.01 mol/L, pH 6.0) and boiled for antigen retrieval. Slides were then blocked with 10% bovine serum albumin in Tris-buffered saline for 30 minutes before incubation in the presence of 1:200 anti-hEAG antibody (Ivivo1 single-chain antibody coupled with alkaline phosphatase) for 18 hours at 4°C in a humid chamber. The slides were washed with a buffer solution and the specific staining reaction was performed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazo- lium as described above.

Keratin Immunocytochemistry. The same general procedure as for EAG1 was used with the following changes: slides were incubated with methanol-hydrogen peroxide (1:100) to block the endogenous peroxidase activity and exposed to primary antibody (anti-keratin Wide Spectrum Screen- ing, Dako, Carpinteria, CA), according to Bellone et al. (18). The slides were washed and incubated with the secondary antibody (biotinylated antirabbit immunoglobulin, DAKO) and subsequently labeled with streptavidin conjugated to peroxidase. 3,3’-diaminobenzidine (Zymed substrate liquid-3,3’-dia-minobenzidine-plus) was applied as chromogen for 5 minutes. Specific reac- tion was observed as brown cytoplasmic staining. Slides were counterstained with hematoxylin. HeLa cells (American Type Culture Collection) expressing keratin were used as positive controls.

Electrophysiology

Whole-cell recordings were acquired from isolated cells with the patch-clamp technique (19) using an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany) and analyzed with Igor Pro (WaveMetrics). Two to three MΩ patch pipettes were obtained by double-pulling Kimax capillaries. Internal solution contained (mmol/L) 140 KCl, 10 EGTA, and 10 HEPES/KOH (pH 7.2). External solution contained (mmol/L) 115 NaCl, 2 CaCl₂, 2 MgCl₂, and 10 HEPES/NaOH (pH 7.2); in some experiments we used free-magnesium solutions or solutions containing 2, 5, or 10 mmol/L MgCl₂. No capacitance compensation was performed. Holding potential was ~80 mV, unless indicated. Experiments were performed at room temperature (20°C to 22°C).

Nature of the Cells from Cervical Cancer Primary Cultures. We obtained 5 primary cultures from the cervical cancer human biopsies. To know if the epithelial and cancerous nature of the sample was preserved in the primary cultures, we looked for epithelial mark- ers with an antikeratin antibody, calculated the percentage of keratin- positive cells, and investigated the expression of the E7 gene from human papilloma virus 16. Fig. 1A shows keratin-positive cells in a cervical cancer primary culture; brown staining reveals the presence of keratin. No staining was seen when the cells were incubated only with the secondary antibody (Fig. 1B). Percentage of epithelial cells per field studied in different cultures was up to 80% (data not shown).

E7 gene expression from Human papilloma virus -16 was studied in three cervical cancer primary cultures. Fig. 1C shows the amplification with specific primers of the E7 gene (lanes 2–4); CaSkI cells expressing the E7 gene were used as positive control (lane 1). Taken together, these results strongly suggest the presence of epithelial cancer cells in the cervical cancer primary cultures.

Effect Expression in Cancerous and Healthy Cervixes. Eag expression was studied by RT-PCR and Southern blot analysis in 5 primary cultures from cervical cancer biopsies, in 1 fresh cervical cancer tissue, and in 12 noncancerous biopsies from normal cervixes.
Fig. 2A shows EAG gene expression in 100% of the primary cultures from cervical cancer (Lanes 1–5). It is worth mentioning that in a patient who was submitted to hysterectomy without any previous evidence of cervical malignancy (negative pap smears), postsurgery pathological studies showed an unexpected endocervical adenocarcinoma expressing eag. Hence, because this eag expression was found in a cancerous tissue, it was grouped together with the samples from primary cultures of cancer cells (Fig. 2A, lane 6).

Studies of eag expression in control cervical biopsies displayed samples either negative or positive for eag, despite all of them coming from patients with negative pap smears. Southern blot experiments from control cervical tissue negative for eag are also shown in Fig. 2A (Lanes 7–12); 8 of 12 samples were negative for eag (only 6 are shown).

Eag expression was observed in 4 control biopsies of normal cervical tissue. Interestingly, 1 of these control eag-positive samples (Fig. 2B, lane 14) came from a patient with human papilloma virus infection, the most important etiological factor associated with cervical cancer. Two other patients in whom eag expression was found in normal cervix presented atypical adenomatous hyperplasia of the endometrium in 1 case and paratubalcystadenoma without atypical cells in the other (Fig. 2B, lanes 15 and 16, respectively). In 1 patient of the eag-positive control samples (Fig. 2B, lane 17) the endometrium was reported as histologically lysed, so a diagnosis could not be determined. hEAG-transfected CHO cells were used as positive control (Fig. 2, lanes 13 and 18).

RT-PCR product identity was determined by nucleotide sequence (data not shown). The amplified products were identical to the sequence reported for hEAG1 (9). We determined that the cells from the cancerous biopsies studied herein express the two different mRNA spliced variants reported for hEAG1 gene (9).

**EAG Channel-Protein in Cervical Cancer Preparations.** EAG at the protein level was confirmed by using anti-hEAG1–specific antibodies in cells from the cervical cancer primary cultures and tissue sections from cervical cancer biopsies from the same patients. As a positive control, EAG channel expression in CHO cells transfected with hEAG1 is shown in Fig. 3A; an almost continuous green-blue immunostaining is observed along the plasma membrane indicated by arrowheads. EAG immunocytochemistry in the cervical cancer primary cultures is shown in Fig. 3B. Again, a continuous green-blue immunostaining indicated by the arrowheads at some points is present in the cell at the left. Finally, EAG channel expression was studied in cervical cancer tissue sections from the same patients. Fig. 3C shows EAG channel expression as a green-blue immunostaining surrounding almost all of the cells; for illustration purposes, staining is indicated by arrowheads at some points. No staining was seen when the

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**Fig. 1. Nature of the cells from the cervical cancer primary cultures.** Immunocytochemistry shows keratin expression (brown staining, A) as an epithelial marker, and up to 80% of the cells expressed keratin; negative control is shown in B. E7 gene amplification by PCR (117-bp product) in three cultures studied (C, lanes 2–4); CaSkI cells were used as positive control (C, lane 1).

**Fig. 2. EAG expression in cancerous and normal cervix.** Southern blot analysis of 475-bp hEAG RT-PCR products is shown for RNAs obtained from primary cultures of cervical cancer biopsies (A, lanes 1–5), endocervical adenocarcinoma (A, lane 6), and control cervix (A, lanes 7–12). eag signals were detected in control cervixes (B) from patients whose samples were diagnosed as human papilloma virus infection (lane 14), atypical adenomatous hyperplasia of the endometrium (lane 15), and paratubalcystadenoma without atypical cells (lane 16); in 1 case (lane 17) it was not possible to establish a detailed diagnosis, because the endometrium was reported as histologically lysed. hEAG-transfected CHO cells were used for positive control (lanes 13 and 18). Hybridization with a cyclophilin (Cyc) probe from the same RNAs is shown at the bottom of each gel.
cells or tissue sections were incubated without the specific antibody (Fig. 3A, inset; Fig. 3B, right photograph; Fig. 3D).

**Current to Voltage (I-V) Relationships in Tumor Cells.** Whole-cell patch clamp experiments were performed in 5 primary cultures from cervical cancer cells. Before exploring EAG activity, we applied voltage ramp protocols (from $-80$ mV to $+120$ mV) to study the current to voltage relationship. Four different shapes of I-V curves in every culture were found (Fig. 4). Some cells (Fig. 4A) displayed clear inward currents from $-30$ mV to $+10$ mV, probably mediated through sodium or calcium channels, followed by an outward noninactivating current. Other cells showed a small outward current at very negative potentials (Fig. 4B) followed by a small inward current near $-25$ mV then followed by an inactivating or rectifying outward current. Fig. 4C shows an I-V curve with a very small inward current at $-50$ mV then followed by an inactivating or rectifying outward current. Cells showing such I-V curve had the highest current density and were the only cells where we detected EAG activity.

**EAG Mediated Currents in Tumor Cells.** We looked for EAG activity in tumor cells by studying their voltage and magnesium-dependent activation. Very negative prepulses have an especially strong effect on EAG activation; the more negative the prepulse potential, the slower the EAG activation (Cole-Moore shift, ref. 20); similarly, the higher the extracellular magnesium concentration, the slower the EAG activation. Fig. 5A shows the potential dependent activation of the outward currents recorded in cervical cancer cells. Unsubtracted traces of currents elicited at $+60$ mV preceded by pulses at different potentials are shown on the left. Prepulse values are indicated for each pulse in magnified traces on the right; the more negative the prepulse, the slower the channel activation.

Magnesium-dependent activation is shown in Fig. 5B. Outward currents elicited at $+60$ mV and preceded by a $-140$ or $-60$ mV prepulse were obtained either in the free-magnesium external solutions (left traces) or in solutions containing 10 mmol/L magnesium.
As expected for EAG, activation is clearly delayed in the presence of magnesium, the effect being more pronounced by applying a very negative prepulse. Fig. 5C shows the required time to reach 80% of the maximum outward current amplitude at different prepulse voltages and extracellular magnesium concentrations. Time to 80% values is bigger at higher magnesium concentrations and very negative prepulses as described for EAG channels (20).

**DISCUSSION**

Because of their oncogenic properties, their restricted distribution in normal tissue and ubiquitous expression in tumor cells (4, 9, 10), EAG potassium channels have gained interest as research tools for cancer detection and therapy.

Cells of our primary cultures from the cervical cancer human biopsies express keratin (up to 80% of the cells in the cultures) and the E7 gene from human papilloma virus 16. Keratin expression can be found both in normal and cancerous cells (18), as well as human papilloma virus 16; however, considering that the primary cultures were obtained from biopsies identified as cancerous tissue, the whole results strongly suggest the presence of epithelial cancer cells in the cervical cancer primary cultures.

Our results of eag expression in all of the cervical cancer samples strongly suggest that eag should be considered as a marker for cervical cancer. As earlier mentioned, in a patient who was submitted to hysterectomy without any previous evidence of cervical malignancy (negative pap smears), post-surgery pathological studies showed an unexpected endocervical adenocarcinoma expressing eag. This case, although unique in this study, emphasizes the potential significance of eag as a tumor marker.

Two alternatively spliced variants have been described for hEAG1 (a and b). With low expositions, Southern blot analysis of all of the eag-positive samples suggest the amplification of two different PCR products (data not shown), one of them corresponding in size with the positive control. Amplifications of each excised band from the gel and sequence confirmed the presence of two different mRNA splice variants (data not shown).

A very important question to answer is whether there is a correlation with the disease stage and EAG expression. Individual variations between patients makes it difficult to address this question with a few samples; we are obtaining more cancer samples biopsies and starting quantitative EAG expression studies to address this important issue.

Although all of the control cervical samples came from patients with negative pap smears, we also found eag expression in 4 of these biopsies. Quite interestingly, diagnosis of 1 of these samples was correlated with the most important etiological factor for cervical cancer, namely, human papilloma virus infection. A plausible scenario is that the increase of eag expression in normal cervix could be an early sign of tumor development.
Another patient in which eag expression was found in normal cervix presented atypical adenomatous hyperplasia of the endometrium, a predisposing condition of endometrial cancer.

In other control eag-positive case, histopathological studies reported the presence of endometrial glands into myometrial smooth muscle (adenomyosis) and, interestingly, a paratubarian serous cystadenoma, a benign epithelial ovarian tumor. In the last patient of the eag-positive control samples the endometrium was reported as histologically lysed; therefore, a diagnosis could not be determined.

More studies are needed to determine whether eag expression might be an early marker for cervical cancer and also an additional tool to diagnose other gynecological disorders related to cell proliferation. We are running trials to study eag expression in dysplastic samples.

Despite major EAG mRNA expression in normal brain (9), EAG-mediated currents in normal tissues have been described only in myoblast before fusion (6), so a defined role for EAG channel in brain still remains to be elucidated. In addition, EAG-mediated currents have been described in some tumor cell lines including SHSY-5Y, IGR1, and MCF-7 from neuroblastoma, melanoma, and breast tumor, respectively (4, 10, 11). Furthermore, eag is expressed in the tumor cell line HeLa (9); however, no EAG-mediated currents have been described in these cells. Here we show for the first time EAG channel activity in human tumors.

We observed four different I-V relationships in every primary culture from cervical cancer. EAG activity was associated exclusively with I-V curves displaying outward noninactivating currents. In the rest of the I-V curves we did not find EAG-mediated currents.

Several reports have shown that EAG currents are modulated through the cell cycle and by cytoskeletal elements (2, 7, 11). Inward currents probably mediated through calcium channels were also recorded in some cells lacking EAG activity. Because our experiments (Fig. 1) strongly suggest the presence of cancer epithelial cells in our cultures, we assume that I-V curve relationship diversity might be explained by different cell cycle stages of the cells in the primary culture. Nevertheless, we could not rule out completely the possible heterogeneity of particular cultures studied for recordings. More studies are needed to determine the nature of the inward currents, their possible involvement in cell proliferation, and the cell-cycle stage of cells displaying EAG-mediated currents.

Because EAG expression and channel activity have been suggested to be important for cell proliferation (9, 12, 13), our findings strongly support the idea of considering EAG as a tumor marker as well as a potential membrane therapeutic target for cervical cancer.

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