Potential Role of the Human Ha-ras Oncogene in the Inhibition of Gap Junctional Intercellular Communication

Mohamed H. El-Fouly, James E. Trosko, Chia-Cheng Chang, and Stephen T. Warren

Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, East Lansing, Michigan (MHEF-JET, C-CC), Departments of Biochemistry and Pediatrics, Division of Medical Genetics, Emory University School of Medicine, Atlanta, Georgia (STW)

The modulation of gap junctional intercellular communication (GJIC) plays an important role during tumor promotion. Several tumor-promoting agents are known to inhibit this form of cellular coupling. In addition, tumor cells and cells expressing certain oncogenic products have been shown to exhibit inhibited or reduced GJIC. The Ha-ras oncogene is expressed in a wide variety of human tumors from different tissues. Its p21 product is a membrane-bound polypeptide, the function of which is not fully characterized. We tested the effects of the expression of the human c-Ha-ras-1 oncogene, derived from the EJ/T4 bladder carcinoma cell line, or the ability of the Chinese hamster V79 cells to conduct gap junctional communication. The junctional competence was studied by two different methods, the scrape-loading/dye transfer technique and the metabolic cooperation assay. The results indicate a strong correlation between the expression of p21 ras protein and the inhibition of gap junctional function. Assuming that reversible inhibition of intercellular communication plays a role during tumor promotion and stable inhibition during the tumor progression phase of carcinogenesis, our data suggest that, while chemical tumor promoters and the ras oncogenes might work by different biochemical mechanisms, they both affect a critical cellular function; namely, GJIC.

Key words: gap junctions, cell-cell communication, Ha-ras oncogene

INTRODUCTION

One form of intercellular communication is mediated via membrane gap junctions, which allow the exchange of ions, nutrients, and regulatory molecules of up to 1.0–1.5 kDa [1]. This coupling mechanism has been implicated in the regulation of tissue homeostasis, cell growth, and differentiation, as well as in synchronization of tissue reactions to stimuli and tissue regeneration [2–4]. Gap junctional intercellular communication (GJIC) was found to be inhibited or reduced in different tumor cells and in cells expressing certain oncogenes or viral products with oncogenic potential (i.e., polyoma middle T antigen) [2, 5–12]. Members of the ras oncogene family encode for guanosine triphosphate binding protein of 21 kDa known as p21 [13], the function of which is not fully understood. Recent evidence indicates that the normal or oncogenic ras p21 protein may be an integral component of a membrane signal transduction system modulating levels of phosphorylatedinositol 4,5 phosphate (PIP2) and its catabolites [14]. Increased levels of sn-1,2-diacylglycerol (DAG), one of the PIP2 byproducts, is known to activate protein kinase C (PKC) [15, 16], a step believed to be critical in cell transformation by ras [14–16]. PKC activation is also induced by tumor promoters [17, 18], such as phorbol esters, which has been reported to cause growth abnormalities in rat fibroblasts [19], and correlates with the inhibition of GJIC [20–22].

The potent tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) may substitute for DAG in the activation of PKC [15], and both TPA and DAG are known to inhibit GJIC in different cell types [23–25]. This, in addition to an observed synergism between TPA and the ras oncogene in vitro transformation [26], suggested a possible similarity in the cellular effects of TPA and the ras gene product. To explore a potential functional correlation between the expression of Ha-ras p21 and intercellular communication, we tested the ability of mammalian cells to perform GJIC, both before and after the introduction of the human c-Ha-ras-1 oncogene.

METHODS AND RESULTS

Chinese hamster lung fibroblasts (V79 cells) were transfected with a hybrid plasmid, pSV2O4, carrying the c-Ha-ras-1 from EJ/T4 bladder carcinoma cell line [27] inserted into the BamHI site of pSV2G [28]. Transfected cells were selected for their resistance to the antibiotic G418. The presence of Ha-ras was verified by Southern blot, using the 6.6-kb BamHI fragment of pSV2O4 plasmid as a probe (data not shown). The expression of p21 was demonstrated by indirect immunofluorescence using rat immunoglobulin G antibodies (IgG) anti-ras (clone Y13-259) [28] and goat anti-rat IgG FITC conjugate. High immunoreactivity to p21 ras protein was detected in the cytoplasm and plasma membrane of the cells transfected with Ha-ras (Figure 1).
Figure 1. Detection of p21 oncoprotein product by indirect immunofluorescence. Cells were fixed in 5% acetic acid in ethanol for 15 min at -20°C, then air-dried and incubated at 37°C for 60 min with 20 μg/ml anti-p21 monoclonal antibody, clone Y13-259 (Oncogene Science, Inc., Mineola, NY). Cells were then washed in PBS and reincubated at 37°C with goat anti-rat IgG FITC conjugate (Sigma Chemical Co., St. Louis, MO) for 30-45 min in a dark, moist chamber. The cells were then washed in PBS and examined under epifluorescence microscopy. The wild-type and cells transfected with pSV2neo reflected a very faint fluorescence (not faithfully reproduced by photography) indicative of the expression of the intrinsic cellular p21. Under identical conditions, the cells transfected with pSV2neo containing c-Ha-ras-1 oncoprotein showed high immunoreactivity in the cytoplasm and plasma membranes. (A) Wild-type V79 cells; (B) V79 cells with pSV2neo; (C) V79 cells transfected with pSV2neo containing c-Ha-ras-1 oncoprotein.

The ability of the cells grown in monolayer cultures to conduct GHG was studied by two different methods, the metabolic cooperation assay [29] and the scrape-loading/dye transfer technique [30]. In the first assay, several clones of 6-thioguanine-resistant (6-TG) cells were obtained from the 6-thioguanine-sensitive (6-TG) wild-type and c-Ha-ras-1 transfected cells by x-irradiation and isolated in the presence of 6-TG. Cocultures of various combinations of 6-TG (HPRT+/wild-type, HPRT-/ras) with 6-TG cell (HPRT+/wild-type, HPRT-/ras) were set and tested for recovery of resistant clones in the presence of 6-TG. In the metabolic cooperation assay, a correlation between the inhibi
HA-RAS IN GJC INHIBITION

The metabolic cooperation assay was utilized as previously described [29]. Briefly, 10^5 HPRT- (6-TG') cells were cocultured with 100 HPRT- (6-TG') cells in 60-mm plastic dishes in the presence of 6-TG. HPRT- (6-TG') cells were obtained by exposing the wild-type V79 cells and each of the six randomly selected c-Ha-ras-1-transfected clones (pmax 1,2,5,6,7, and 20) to a total of 1000 rad of X-rays. The putative HPRT- mutants were then selected in 10 \mu M 6-TG. The control series (open bars) included six randomly selected V79 clones (C1-6), to verify the homogeneity of GJC in the wild-type cells. In addition to the wild-type (CO) and pSV2neo transfected cells (N1-3). Each of the six ras-transfected 8-TG' clones was cocultured with the 6-TG' cells derived from the wild-type V79 cells (hatched bars). In addition, three randomly selected HPRT- subclones derived from each of the ras-transfected cells were cocultured with their respective parental 6-TG' cells (solid bars). Cocultured combinations (10 plates each) were incubated in Dulbecco's modified medium at 37°C in humidified air with 5% CO_2 for 7-10 d. 6-TG' colonies were then fixed, stained, and scored, and Student's t-test was utilized for statistical analysis. A significant increase or decrease in 6-TG' colony recovery was detected in cells expressing the introduced c-Ha-ras-1 oncogene (in all experiments or cells expressing p21, P> 0.00001).

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Figure 3. Detection of GJIC by scrape-loading/dye transfer method. As previously described [30], this assay monitors the transfer of the membrane-impermeable fluorescent dye LY (m.w. 457.2) via gap junctions from primary-loaded cells into contiguous ones. Cells were plated in 35-mm dishes overnight to confluency at 37°C (6–8 plates/cell line or clone). Before scrape-loading, the cells were rinsed one or four times with PBS, drained, covered with 2 ml of 0.05–0.1% LY, dissolved in PBS at room temperature, and immediately scraped with a wooden probe or a sharp blade. The dye was left on the cells for 60–90 s, then discarded, and the cells were rinsed several times with PBS. Fresh medium was added and the cells were examined under epifluorescence microscopy (Nikon Diaphot phase-contrast with Orak HBO 100 W UV light attachment; Nikon Inc., Garden City, NY). Equal surface areas, equivalent to 20 x objective lens field, were compared, and fluorescent cells were counted visually. An average of 100 cells were present in each randomly selected field. (A) Positive LY dye transfer in control cells not transfected with c-Ha-ras-1 oncogene; (B) absent dye transfer in V79 cells expressing c-Ha-ras-1 oncogene.


