Tyrosine-kinase expression profiles in human gastric cancer cell lines and their modulations with retinoic acids

It is estimated that there are 1000–2000 different protein kinases in the human genome (Hunter and Cooper, 1985; Robinson et al., 2000). Less than 10% of all kinases are protein tyrosine kinases (PTK), but many tyrosine kinases are involved in growth signalling, and alterations of these kinases often result in cellular transformation (Hunter and Cooper, 1985). Many PTK genes are proto-oncogenes and some of them are associated with human cancer progression, including erbB2/neu in breast cancer and met in gastric cancer (Kameda et al., 1990; Kuniyasu et al., 1992). These genes are highly conserved from nematode to human and are involved in important biological functions such as growth, differentiation, development, apoptosis and signal transduction. On the basis of their cellular localisation, these PTKs can be further classified as receptor-type or nonreceptor-type PTK and dual kinases that can phosphorylate both tyrosine and serine/threonine residues. Since PTKs share significant homologies in their kinase catalytic domain, degenerated polymerase chain reaction (PCR) primers can be designed according to the amino-acid sequence submotifs of the kinase catalytic domain (Robinson et al., 1996; Lin et al., 1998). In previous studies, we identified 25–35 different PTK genes expressed in cells by simple reverse transcriptase–polymerase chain reaction (RT–PCR) reaction (Lin et al., 1998, 1999; Wu et al., 2000). Since then, we implemented an improved restriction analysis of gene expression (RAGE) profiling approach, developed at Dr Hsing-Jien Kung’s laboratory (Robinson et al., 1996), which utilises restriction enzyme digestion and gel electrophoresis for quick and efficient kinase profiling (manuscript in preparation). Recently, this profiling method has been used to generate expression profiles of human breast cancer cell lines (Meric et al., 2002) and also identified axl receptor tyrosine kinase as one of the adenovirus E1A target genes in ovarian cancer cells (Lee et al., 1999).

Retinoic acid (RA) and its metabolites, the retinoids, are required for differentiation and tissue maintenance. Deficiencies in the metabolism of retinoids are associated with severe defects of vertebrate embryonic development (Gudas, 1992; Chambon, 1994). This reagent has been used in the treatment of acute promyelocytic leukaemia with good success (Huang et al., 1988), and recently it was shown to suppress the growth of gastric cancer cell lines (Shyu et al., 1995; Naka et al., 1997). The RA signalling pathway involves specific high-affinity receptors, which belong to ligand–inducible transcription factors (Chambon, 1996). The RA ligand-receptor complexes modulate target genes’ expression by binding to the retinoid-responsive elements in the promoter regions. Among PTKs, c-erbB2/neu promoter contains retinoid-responsive elements, which are weakly modulated by the addition of RA in the medium (Hudson et al., 1990). All-trans-RA was recently shown to enhance mRNA expression of c-fms receptor tyrosine kinase in human breast carcinoma cells (Sapi et al., 1999). Extensive research on the expression of PTKs and RA modulation is lacking and details regarding the mechanisms involved in the RA-regulated growth inhibition of gastric cancer cells are not known. In the present study, we examined the overall PTK expression profiles in gastric cancer cells treated with 9-cis-RA or all-trans-RA. We found that expressions of eph (ephA1) and hek5 (ephB2) were upregulated following treatment in several gastric cancer cell lines.

Keywords: protein-tyrosine kinase; retinoic acid; gene expression profiles
MATERIALS AND METHODS

RT–PCR amplification and RAGE profiling of human tissues

Poly(A) selected mRNA were purchased from Clontech, including eight adult tissues (brain, kidney, liver, lung, pancreas, placenta, small intestine and stomach) and four foetal tissues (foetal brain, foetal kidney, foetal liver and foetal lung). Reverse transcription was carried out with a cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). The PCR primers are derived from the conserved motifs DFG and DVV of tyrosine-kinase catalytic domain as described (Meric et al., 2002). Several pairs of degenerated PCR primers were designed, which would expect a PCR product of 150–170 bp range. In order to identify the restriction-digested fragments, the S'-sense primers were radio-labelled with 33P by polynucleotide kinase in this RAGE method. The PCR reactions were conducted at 42°C annealing temperature for five cycles and then at 55°C for 25 cycles with an Accugen 9600 PCR thermocycler and Taq polymerase (GIBCO-BRL, Rockville, MD, USA). The final PCR products at 170 bp fragment products were separated and eluted from agarose gels. An equal amount (20,000 c.p.m.) of the eluted PTK ampiclon was then digested with respective restriction enzymes (New England BioLabs, Beverly, MA, USA) and analysed with denaturing 7% sequencing polyacylamide gels. Following electrophoresis, the gel was dried and exposed to an X-ray film or processed by a Fuji BAS documentation system. The resulting restriction-digested fragments, the S'-sense primers were radio-labelled with 33P by polynucleotide kinase in this RAGE method. The PCR reactions were conducted at 42°C annealing temperature for five cycles and then at 55°C for 25 cycles with an Accugen 9600 PCR thermocycler and Taq polymerase (GIBCO-BRL, Rockville, MD, USA). The final PCR products at 170 bp fragment products were separated and eluted from agarose gels. An equal amount (20,000 c.p.m.) of the eluted PTK ampiclon was then digested with respective restriction enzymes (New England BioLabs, Beverly, MA, USA) and analysed with denaturing 7% sequencing polyacylamide gels. Following electrophoresis, the gel was dried and exposed to an X-ray film or processed by a Fuji BAS documentation system. The expected sizes of PCR products were 371 bp for GAPDH, 318 bp for yes and 420 bp for hek5. The final products were analysed in 2% agarose gel, visualised by ethidium bromide staining and recorded with the Alpha Innotech IS-500 gel documentation system.

Western blot analysis of yes kinase

Gastrointestinal cancer cell lines were incubated in NP-40 lysis buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 0.25% SDS, 1 mM sodium vanadate, 1 μg ml−1 protease inhibitors, 200 μg ml−1 chymostatin and 1 mM PMSF for 30 min at 4°C. Cell lysates were then centrifuged at 14,000 rpm for 10 min and supernatants were harvested. A measure of 100 μg of lysates was boiled and electrophoresed in 7.5% SDS–polyacrylamide gels under reducing conditions. The separated proteins were then electrophoretically transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). Following blocking by blocking solution of 10% nonfat dried milk for 1 h at room temperature, the membrane was blotted with anti-yes-specific antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) or anti-β-actin antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) at room temperature for 1 h. Following washing with Tris-buffered saline with 0.05% Tween-20 and incubation with horseradish peroxidase-conjugated secondary antibodies, protein bands were detected by the enhanced chemiluminescence method (Amersham Life Science, Piscataway, NJ, USA).

RESULTS

By using degenerated primers from submotifs VII and IX of the kinase domain as well as RT–PCR, we were able to easily amplify PTK genes expressed in cells. In addition to the previous ‘cloning and sequencing’ method, we utilised an improved RAGE method to provide a more efficient, economical and expeditious tyrosine-kinase profiling approach. We first established the PTK RAGE protocol in our laboratory by using a panel of human tissues. Samples from 12 tissues could be displayed on a single sequencing gel; thus we could effectively screen all known human PTKs in a gel; thus we could effectively screen all known human PTKs in a single sequencing gel; thus we could effectively screen all known human PTKs in a single sequencing gel; thus we could effectively screen all known human PTKs in a single sequencing gel; thus we could effectively screen all known human PTKs in a single sequencing gel; thus we could effectively screen all known human PTKs in a single sequencing gel; thus we could effectively screen all known human PTKs in a single sequencing gel.
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represented by similar size restriction fragments, and alternative restriction enzymes would be necessary to identify single-gene-specific restriction fragments. It was calculated that just 15–20 restriction enzymes (four-base or five-base hitters) were required to cover all known PTKs. Among these kinases in Figure 1, ron kinase expression was found in selected tissues only (brain, lung, pancreas, small intestine, stomach, foetal brain and foetal lung). This finding correlated with the reported in situ hybridisation and Northern blot data (Quantin et al., 1995), which validated our PTK profiles. By eliminating cloning and sequencing steps in previous profiling approaches (Lin et al., 1998) and adopting end-labelled PCR primers, this RAGE method is more quantitative as the intensity of each PTK fragments could reflect the PTK molecules expressed in cells.

This approach enabled us to establish comprehensive PTK expression profiles from a large number of samples in a single experiment, and thus we were able to generate comprehensive PTK profiles from six different human gastric cancer cell lines in a very limited amount of time. Two cell lines (AGS and SC-M1) were derived from primary tumours and others (HR, Kato III, NUGC, SC-M1 and TSGH) were derived from metastatic tumours from various organs. With this PTK profile information, we hoped to learn more about the biological significance of particular PTKs in the oncogenesis of these human gastric cancer cells.

As shown in Figure 2, the amplified PTK products were digested with Hae III restriction enzyme and displayed on a sequencing gel. Samples from six human gastric cancer cell lines could be displayed on a single sequencing gel; thus, we could effectively screen all known PTKs with only a few gel displays. In Figure 2, several PTKs could be identified by their specific restriction fragment sizes. Alternative restriction enzymes would be necessary to identify all PTKs according to their unique specific restriction fragment. This RAGE method is more quantitative as the radioactive isotope is labelled on the primers. Therefore, we could measure PTK expression levels by enumerating radioactivities of each fragment. In Figure 3, we correlated the expression of the yes PTK gene by specific primers. The RT–PCR results reflected the RAGE profile. AGS and Kato III showed a lower level of yes expression in both assays. In order to validate the protein expression level, Western blot analysis was performed with yes PTK-specific antibody. As shown in Figure 4, overexpression of yes tyrosine-kinase protein is evident. The yes PTK expression...
pattern is similar in the mRNA and protein level in this study (Figures 3 and 4).

Figure 5 summarises the complete PTK profiles in these gastric cancer cell lines, and a total of 39 protein kinases were identified. Each cell line generated a unique and distinct PTK expression profile. This valuable information can now be used as an overall PTK expression reference database and serve as PTK fingerprints of different gastric cancer cell lines. Four cell lines of Asian origins (Japan and Taiwan) presented surprisingly similar profiles, although Kato III had an enhanced expression on brk and cak kinases and a lower expression level on slk. On the other hand, two PTKs (hek8 and mer) expressed at a higher level in AGS and HR cells. There were also significant variations between cell lines, including a low expression level of ron in HR cells. This information allows us to discern the tyrosine-kinase expression pattern in a particular cell line and to select proper cells for tyrosine-kinase functional studies. For example, AGS might not be suitable for erbB2/neu transfection experiments, since it has the highest expression of that particular tyrosine kinase (Figure 5). On the same token, HR cells would be the preferable choice for studying cak kinase because of its extremely low expression level (Figures 2 and 5). There was a small number of different PTKs identified between previous cloning experiments using HR cells and the new RAGE profile of HR cells. This could largely be attributed to the different degenerated primers used in the two types of experiments.

Upregulation of some PTKs, such as c-erbB2/neu, confers resistance to chemotherapeutic reagents (Yu et al, 1996). It is important to know the PTKs modulated by chemotherapeutic reagents and possible resistant mechanisms involved in cancer cells. This RAGE-based PTK profiling allowed us to quickly screen tyrosine-kinase genes modulated in cells treated with various reagents. We first selected RA for our studies, since it directly modulates gene expressions following its binding to RAR or RXR receptors. In addition, RA was shown to suppress gastric cancer cell growth in vitro (Shyu et al, 1995; Naka et al, 1997). We used both 9-cis-RA and all-trans-RA and three RA responsive cell lines (NUGC, SC-M1, TSGH) in this study. By MTT assay, we had determined that growth of these gastric cancer cells was inhibited by $10^{-8} - 10^{-6}$ M in both all-trans-RA and 9-cis-RA. Cell death was observed after 5–7 day treatment with RA. Since it was shown that most RA responsive genes are activated by 24 h posttreatment (Bouillet et al, 1995), we therefore selected an optimum condition of $10^{-6}$ M under serum-free conditions and a 36-h treatment schedule for mRNA extractions, at which point most cells (> 80%) were still viable.
According to PTK RAGE profile analysis, there were several kinases upregulated by 9-cis-RA and all-trans-RA in all cells, including eph, hek5 and several novel kinases. In Figure 6, hek5 RAGE profiles are shown in the upper panel. To verify the expression of hek5, we also performed RT–PCR experiments with hek5-specific primers (Figure 6, lower panel). Similar results were obtained with the eph kinase (not shown). There was a notable increase in hek5 expression level in SC-M1 and TSGH cells (Figure 6). Both eph and hek5 belong to the eph tyrosine-kinase subgroup, which is the largest subgroup of tyrosine kinases and which plays important roles in development (Zisch and Pasqualle, 1997). Hek5 expression was previously shown to be elevated in human gastric cancer tissue (Kiyokawa et al, 1994). However, the present study is the first to show the modulation of eph and hek5 by RA treatment in human gastric cancer cell lines. In a reported RA-resistant cell line (AGS) (Shyu et al, 1995), basal levels of hek5 and c-erbB2/neu expression were markedly elevated in comparison with those of RA-sensitive cell lines (Figure 6). Since amplification of c-erbB2/neu could lead to drug-resistant phenotypes in tumour cells (Yu et al, 1996), it would be interesting to investigate if hek5 kinase is also involved in such mechanisms.

**DISCUSSION**

We have developed a simple, economical and efficient method for generating comprehensive expression profiles of gene families. With this technique, we were able to effectively generate PTK expression profiles of human gastric cancer cell lines. These profiles serve as fingerprints for each tumour cell line and are extremely valuable in correlating various biological properties of these tumour cells in vitro and in vivo. Thus, we should be able to learn more about the roles of PTKs in tumour initiation and progression, and develop better therapeutic reagents or strategies.

Retinoic acid is the active derivative of vitamin A, and interacts with two classes of receptors (RARs and RXRs). Both RARs and RXRs are ligand-dependent transcription factor receptors (Chambon, 1996). RAR and RXR families each possess three receptor subtypes (α, β and γ), which have been demonstrated to be differentially expressed in various tissues during the mouse embryo development (Ruberte et al, 1991). RARs are activated by all-trans-RA and 9-cis-RA, whereas RXRs are only activated by 9-cis-RA (Heyman et al, 1992). RA regulates the growth and differentiation of many different cell types, and plays an important role in development (De Luca, 1991). Many genes are known to be modulated by RA treatment, such as Stra genes (Strα-13) (Bouillet et al, 1995; Bouddjelal et al, 1997), midkine (Muramatsu et al, 1993), ICAM-1 (Cilenti et al, 1995), STAT1 (Kolla et al, 1996), IL-8 (Harant et al, 1996) and IL-2 receptor alpha subunit (Bhatti and Sidell, 1994). Among signal transduction molecules, c-erbB2 promoter activity and X17C (a novel MAP kinase phosphatase) were found to be upregulated by RA (Hudson et al, 1990; Mason et al, 1996). RAR and RXR can also be phosphorylated by protein kinases (Tahayato et al, 1993). In a previous report on RARs' and RXRs' Northern mRNA expression level of gastric cancer cell lines (Shyu et al, 1995), enhanced expression of RARβ2/RARγ was observed in SC-M1 cells and enhanced RARβ level was found in TSGH cells. Both RA-sensitive cell lines (SC-M1 and TSGH) had also increased RXRβ expression, but only TSGH had higher RXRβ expression transcript. The authors implicated that RARβ and RXRβ may not be related to RA-mediated growth inhibition in these sensitive cells, while RARα may play an important role in these cells (Shyu et al, 1995). Nevertheless, hypermethylation of the RARβ promoter resulted in the loss of RARβ, and nm23-H1 genes were reported in diffuse-type gastric cancers (Oue et al, 2002). With our PTK profiling technique, we identified two PTKs (eph and hek5) that also responded to RA treatment in RA-sensitive gastric cancer cell lines. The actual roles of these kinases in RA-mediated growth inhibition need to be further examined. Owing to the complex homo- or hetero-dimer interactions between RARs and RXRs, it is difficult to dissect the detail mechanisms of RARs' and RXRs' activation, not to mention mechanisms involving other retinoic/retinol binding proteins within the cytoplasm. It is also important to identify those novel kinases in RAGE profiles that are regulated by RA treatments. This could be achieved by direct cloning of restriction fragments from gels or by bioinformatic databases interrogations. We did not observe the expected upregulation of c-erbB2/neu PTK in the gastric cancer cells examined.

Eph receptor tyrosine kinases (including eph and hek5) and their ligands (ephrins) play important functions during embryogenesis, especially in neuronal tissue growth, migration and differentiation (Zisch and Pasqualle, 1997; Mellitzer et al, 1999). Overexpressions of ephs and ephrins are documented in many human cancers including gastrointestinal cancers (reviewed in Nakamoto and Bergemann, 2002). There are more than 14 members of the eph PTK family and at least eight different ephrins. Since ephrins are attached to the cell membrane by either glycosylphosphatidylinositol-anchor or a single transmembrane domain, they might act as receptor-like signalling molecules that can be phosphorylated and then transduce signals (Bruckner et al, 1997). Autocrine expression of eph kinase and ephrins was recently demonstrated in human lung cancer cell lines and tissues (Tang et al, 1999). Ephrin-B1 (also known as lerk-2, stra-1 and cek5-L), which is the ligand for hek5, hek10 and elk, is strongly upregulated by RA in mouse P19 embryonal carcinoma cells and D3 embryonic stem cells (Bouillet et al, 1995). Since hek5 is also upregulated in gastric cancer cells following RA treatment, we are now investigating the expression of ephrin-B1 and hek5 in detail to determine their functions in human gastric cancer cell growth and in RA-mediated cell growth inhibition. On the other hand, hek8 (epha4) was downregulated by RA treatment in developing chick limb buds (Patel et al, 1996). However, there is no literature report regarding the RARs' or RXRs' involvement in the eph/ephrin signalling pathway following RA treatment. It is evident that extremely complicated networks exist within the eph/ephrin as well as the RA/RAR-RXX systems because of the large numbers of gene family members and crosstalk interactions. This report provides an initial but significant observation on the crosstalk between these two complicated signal networks. Such crosstalk interactions among different signal transduction pathways have been documented recently between IL-6 and EGF pathways in prostate cancer cells (Qiu et al, 1998). With this new RAGE profiling approach, we are able to show the potential interactions between eph and RA pathways in human gastric cancer cell lines.
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