Stimulation of CEACAM1 expression by 12-O-tetradecanoylphorbol-13-acetate (TPA) and calcium ionophore A23187 in endometrial carcinoma cells

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Downregulation of carcinoembryonic antigen-related cell adhesion molecule (CEACAM1), a cell adhesion molecule with tumor suppressing properties has been observed in a high percentage of carcinomas of the endometrium and other malignancies. The mechanisms for the dysregulation and the role of hormones and cytokines on the expression of CEACAM1 in endometrial carcinomas is unknown. We therefore studied the effect of estradiol, medroxyprogesterone acetate (MPA), RU486, gamma-interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), 12-O-tetradecanoylphorbol-13-acetate (TPA) and calcium ionophore A23187 on the expression in the non-expressing endometrial tumor cell lines Hec1B and Skut1B, respectively. No induction of CEACAM1 expression was observed in Hec1B endometrial adenocarcinoma cells in response to hormones and cytokines whereas treatment with TPA and calcium ionophore A23187 resulted in the strong expression of endogenous CEACAM1 on the mRNA and protein levels. In contrast, no induction of CEACAM1 expression was observed in endometrial mixed mesenchymal Skut1B cells. Studies of other members of the CEACAM family revealed that the re-expression in Hec1B carcinoma cells is restricted to CEACAM1 suggesting a cell type-specific and cell type-independent mechanism of CEACAM1 activation via the protein kinase C (PKC) pathway. Induction of CEACAM1 expression was dependent on protein kinase C protein synthesis and luciferase reporter assays with CEACAM1 promoter constructs demonstrated that the re-expression of CEACAM1 is regulated at the transcriptional level. This is the first report demonstrating that activators of PKC are able to specifically induce the expression of CEACAM1 in human carcinoma cells and our findings may provide a basis for the therapeutic inhibition of tumor growth in malignancies in which CEACAM1 is downregulated.

Introduction

The human carcinoembryonic antigen-related cell adhesion molecule (CEACAM1), the former biliary glycoprotein (BGP) is a member of the carcinoembryonic family which is part of the immunoglobulin superfamily (1–4). CEACAM1 localized on chromosome 19q13.2 is differentially spliced and expressed on the cell surface of many epithelia, granulocytes and on endothelial cells of microvessels in proliferating human tissues (5,6). The highly glycosylated protein has multifunctional properties and is involved in important cellular processes like cell adhesion, differentiation, signal transduction and serves as a microbial receptor (1,2,7–9). Recently, we have demonstrated that CEACAM1 plays an important role in blood vessel formation during angiogenesis (10). Muller et al. (11) suggest that transmembrane CEACAM1 expressed on endothelial cells is implicated in the activation phase of angiogenesis by affecting the cytoskeleton architecture and integrin-mediated signaling. In addition, CEACAM1 modulates T cell activation and thus could facilitate crosstalk between epithelial cells and T lymphocytes in immune response (12–18). It also could be possible that CEACAM1 plays a role in the mediation of cellular apoptosis (19).

Compared with the corresponding normal tissue, altered expression of CEACAM1 has been found in many human tumors. In particular, downregulation of mRNA and protein levels was observed in a high percentage of carcinomas of the endometrium and the loss of expression correlated with the grade of malignancy (20). Decreased levels of expression were also found in adenomas and carcinomas of the colon as well as in breast and liver cancer (21–25). In contrast, increased CEACAM1 expression has been reported for carcinomas of the lung and stomach (26,27). We have also recently shown that CEACAM1 can act to enhance invasiveness in both melanoma (28) and trophoblast tumors (29).

Restoration of CEACAM1 expression in tumor cells of the colon, breast, bladder and prostate resulted in the inhibition of tumor development in animal models (1,30–32). These observations together with the findings in human malignancies indicate that CEACAM1 can act as a tumor suppressor in certain tumors.

The mechanisms and regulatory pathways for the altered expression of CEACAM1 in human tumors are unknown. Induction of the expression of CEACAM family members was observed in colon carcinoma cell lines after stimulation with the cytokines gamma-interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α). Phan et al. (34) investigated the effects of androgen and the androgen receptor on CEACAM1 expression and could find an increased activity of the CEACAM1 promoter. Moreover, activation of the CEACAM1 promoter in response to insulin, dexamethasone or cAMP has been reported for rat hepatoma cells (35). However, the role of hormones and cytokines in the dysregulation of CEACAM1 in tumors is unclear. Studies on the human CEACAM1 promoter lacking typical TATA and CAAT boxes revealed that the upstream regulatory factor, the hepatic nuclear factor 4 (HNF-4) and activating protein 2 (AP-2)-like factors are involved in the regulation of the basal transcription.

Abbreviations: CEACAM1, carcinoembryonic antigen-related cell adhesion molecule; IFN-γ, gamma-interferon; MPA, medroxyprogesterone acetate; PKC, protein kinase C; TNF-α, tumor necrosis factor alpha.
of CEACAM1 (36,37). Muenzner et al. (38,39) could demonstrate that Neisseria gonorrhoeae-triggered CEACAM1 upregulation involves activation of the transcription factor nuclear factor κB (NF-κB) through Toll-like receptor-4 (Toll-R4). Recently Phan et al. (40) identified SP2 as a transcriptional repressor of carcinoembryonic antigen-related CEACAM1 in tumorigenesis. So far, no altered expression of these factors has been reported in human tumors. To gain further insights in the regulation of CEACAM1 in endometrial carcinomas, we investigated the effect of estradiol, medroxyprogesterone acetate (MPA), IFN-γ, TNF-α, TPA and calcium ionophore A 23187 on the expression of CEACAM1 using the human endometrial tumor cell lines Hec1B and Skut1B as a model system.

Materials and methods

Cell culture

Hec1B human endometrial adenocarcinoma cells, endometrial mixed mesenchymal Skut1B cells, SW480 colon carcinoma cells, T47D breast carcinoma cells and CECV304 bladder carcinoma cells were obtained from ATCC (Rockville, MD). HEC1B, SW480, T47D and CECV304 cells were maintained in low glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum, 2 mM l-glutamine and penicillin/streptomycin. Cells were kept at 37°C in a humidified atmosphere at 5% CO2, passaged twice weekly and analyzed when 60–80% confluent. For experiments involving stimulations with steroid hormones, the cells were routinely kept in medium containing charcoal-stripped fetal bovine serum in order to avoid additional exposure to steroids from the medium.

Treatmen, RNA preparation, PCR, northern and western blot analysis

Before analysis, cells were stimulated for 24 h with either estradiol alpha (10−7 M) (Sigma, Berlin, Germany), MPA (2.5 × 10−7 M) (Sigma), RU486 (2.5 × 10−7 M) (Roussel-UCLAF), IFN-γ (100 U/ml) (Boehringer, Mannheim, Germany), TNF-α (500 U/ml) (Boehringer), TPA (10−7 M) (Sigma) or calcium ionophore A23187 (1 μg/ml) (Sigma). Transfection was performed by the lipofection method with 0.4 μl of DNA expression vectors coding for the estrogen, progesterone A and progesterone B receptor, respectively. Actinomycin D and cycloheximide (Merck, Darmstadt, Germany) were added to the culture medium prior to treatment at a final concentration of 5 and 50 μg/ml, respectively.

Total RNA was isolated by direct lysis of the cell monolayer using the Trizol reagent (Gibco, Karlsruhe, Germany). cDNA synthesis was carried out with 250 ng of total RNA with random hexamers applying Superscript II reverse transcriptase under conditions recommended by the supplier (Gibco). PCR amplification was performed with 10 pmoles of each primer in a total primer of 50 μl with Taq-Polymerase (Gibco) on a Biozym thermocycler (Mi Research, Waltham, MA) with initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C, annealing at given temperatures (Table I) and extension at 72°C, for 1 min each with a terminal step of extension at 72°C for 10 min. PCR products (10 μl) were analyzed on 2.5% agarose gels stained with ethidium bromide.

Northern blot analysis was performed with 15 μg of total RNA and hybridized with oligonucleotide probes specific for CEACAM6 and CEACAM1 under conditions described previously (21,22). For the detection of CEACAM1 and CEACAM5, DNA probes were used corresponding to the cytoplasmatic domain of CEACAM1 (231 bp) and the 3′-untranslated region (258 bp) of CEACAM5, respectively. Probes were generated by PCR from normal human colonic cDNA under the conditions given above, gel-purified and radioactively labeled by random priming. Hybridization and rehybridization were carried out as previously described (21,22) except that stringent washes were performed at 55°C for CEACAM1, at 50°C for CEACAM5 and at 60°C for the G3PDH probe (BD Pharmingen, Heidelberg, Germany).

Protein extraction and western blot analysis was performed with 100 μg of protein as described previously (5). Levels of CEA (CEACAM5) in cellular lysates and tissue culture supernatants were directly measured by a routine enzyme immunoassay (Bayer, Leverkusen, Germany).

Luciferase reporter assay

Promoter constructs were generated from the cosmId clone R26908 by digestion with the restriction enzymes XmnI and XhoI. The recovered 2.4 kbp fragment was cloned in pBluescript II SK+ (Pharmacia, Freiburg, Germany) and subcloned after digestion with KpnI and NotI in pGL3-basic (Promega, Amriswil, Switzerland) placing the first 1858 bp of the human CEACM1 promoter upstream of the luciferase gene (P1). Promoter construct P2 representing 624 bp of the upstream regulatory region was generated by digestion of P1 with PstI and subsequent re-ligation of the vector. The promoter regions P3 (376 bp) and P4 (206 bp) were amplified from P1 by PCR (Table I), subcloned in pCR2 (Invitrogen, Karlsruhe, Germany) and cloned after digestion with Xhol and HindIII in pGL3-basic. The integrity of the constructs was confirmed by DNA-sequencing. For the luciferase assay, 2 × 104 cells were plated in triplicate on 100-mm-diameter 12-well tissue culture plates (Costar, UK) and transfected after 24 h by the lipofection method with 1 μg of the reporter plasmid/well. After 24 h, transfected cells were cultivated further for 18 h in the presence or absence of TPA and ionophore A23187. Subsequently, cells were trypsinized, washed, pelleted and lysed with reporter lysis buffer (Promega). After one freeze-thaw cycle, luciferase activity in the lysate was determined using the Lumat LB 9501 Luminometer (Berthold, Pforzheim, Germany). A β-galactosidase control expression vector was co-transfected to normalize for transfection efficiency. Each measurement was performed in triplicate and experiments were repeated at least three times.

Results

Induction of CEACAM1 expression by TPA and ionophore A23187: RT–PCR, northern and western blot analysis

In order to study the regulation of the CEACAM1 gene, different hormones, cytokines and chemical agents were tested for the induction of expression in the human endometrial adenocarcinoma cell line Hec1B. Induction of mRNA expression was analyzed by RT–PCR with CEACAM1 specific primers binding to the A2-domain and the 3′-UTR region, respectively allowing the detection of the long isoform CEACAM1/a (Figure 1). In accordance with previous data, CEACAM1 expression was not detectable by RT–PCR in untreated Hec1B cells (6). No effect on the CEACAM1 mRNA expression was observed after treatment with estradiol, MPA and the progesterone antagonist RU486. As Hec1B cells are progesterone receptor negative, transfected cells expressing the estrogen alpha or progesterone receptor, respectively were analyzed in parallel. No induction was observed after treatment indicating that the CEACAM1 expression in Hec1B cells is not dependent on estradiol or MPA, respectively. In contrast to colon carcinoma cell lines in which stimulation with the cytokines IFN-γ and TNF-α resulted in the upregulation of the CEACAM family members CEACAM1, CEACAM5 and CEACAM6 (33),

<table>
<thead>
<tr>
<th>Primer</th>
<th>T</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>CEACAM1-U</td>
<td>55°C</td>
<td>5′-AAGTAGTGATCTATACCTGCCAGGAC-3′</td>
</tr>
<tr>
<td>CEACAM1-L</td>
<td>60°C</td>
<td>5′-CTCAAGCCTATGATGGGTTT-GATCCA-3′</td>
</tr>
<tr>
<td>NB-CEACAM1-U</td>
<td>52°C</td>
<td>5′-AGGACGACAGGGTTTACATTG-3′</td>
</tr>
<tr>
<td>NB-CEACAM1-L</td>
<td>55°C</td>
<td>5′-GGGTTACGTGTCCTGCTGAC-3′</td>
</tr>
<tr>
<td>NB-CEACAM5-L</td>
<td>58°C</td>
<td>5′-AAGGGGAGGAGGAGGAGG-3′</td>
</tr>
<tr>
<td>P3/4-L</td>
<td>55°C</td>
<td>5′-CTCTCGTCTGCTGAGTTGGT-3′</td>
</tr>
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T, annealing temperature; U, upstream of the start codon; L, downstream of the start codon.
no effect of these cytokines was observed in Hec1B cells. Unexpectedly, strong induction of mRNA expression represented by a PCR product of 878 bp was observed after treatment of Hec1B cells with the phorbol ester TPA and higher levels of induction were obtained in combination with the calcium ionophore A23187. Lower levels of mRNA induction were found when Hec1B cells were separately treated with TPA or ionophore A23187 suggesting that both substances are acting additively on the induction of the expression of CEACAM1 (data not shown).

To further investigate the induction of CEACAM1 expression in Hec1B cells by TPA and ionophore A23187, a time-course experiment was performed and levels of mRNA expression were analyzed by northern blot (Figure 2). Hybridization was carried out with a probe specific to the cytoplasmatic domain allowing the detection of most of the CEACAM1 isoforms. Induction of the long isoform CEACAM1a (3.9 kb) was observed after 6 h increasing to 10- to 20-fold levels after 12 to 48 h of treatment. The second transcript of 1.5 kb representing the isoform CEACAM1c (lacking the 1. exon of the cytoplasmatic domain and the long 3'-untranslated region) was detectable after 12 h of treatment. In contrast to Hec1B cells, no induction of mRNA expression was observed in CEACAM1 negative endometrial mixed mesenchymal Skut1B cells (data not shown) suggesting that the re-expression of CEACAM1 is restricted to endometrial adenocarcinoma cells. We then tested the effect of TPA and ionophore A23187 on the mRNA expression of other members of the CEACAM gene family (Figure 3). In contrast to the strong re-expression of CEACAM1, no induction of CEACAM5, CEACAM6 and CEACAM7 (data not shown) expression was observed in Hec1B cells indicating that TPA and ionophore A23187 are specifically acting on the expression of CEACAM1.

In accordance with the northern blot data, specific induction of CEACAM1 protein expression was observed by western blot analysis (Figure 4). Applying the CEACAM1 specific mAb 4D1/C2 and mAb T84.1 with a broad specificity for members of the CEACAM family.
Identification of regulatory elements in the CEACAM1 promoter

TPA and calcium are known to activate protein kinase C which in turn induces gene transcription through the activation of different transcription factors like AP1, AP2, AP3 or NFκB (42). Comparison with corresponding consensus sequences of transcription factors revealed that several potential binding sites are present in the human CEACAM1 promoter (Figure 6). To further determine the functional significance of these putative binding sites, different parts of the CEACAM1 promoter were placed upstream of the bacterial luciferase gene and the transcriptional activity of transiently transfected constructs was determined before and after treatment with TPA and ionophore A23187 in Hec1B and Skut1B cells, respectively (Figure 6). In comparison with unstimulated Hec1B cells, the construct P1 representing 1858 bp of the CEACAM1 promoter showed a 7- to 8-fold increase of luciferase activity after treatment. Comparable levels of induction were found for P2 indicating that inducible elements are localized in the first 624 bp of the CEACAM1 promoter. Lower levels of luciferase activity with 3- to 4-fold induction above levels of unstimulated Hec1B cells were observed for the shorter promoter fragments P3 and P4, respectively. Therefore, important inducible elements are most likely localized in P2 upstream of P3 corresponding to a promoter region of −377 to −624 bp upstream of the start codon. In accordance with the northern blot data, no significant activation of the CEACAM1 promoter constructs was observed in Skut1B cells after treatment with TPA and ionophore A23187 (Figure 6A). In addition we investigated the breast carcinoma cell line T47D and the colon carcinoma cell line SW480. These two cell lines showed lower activity levels of luciferase in comparison to Hec1B cells. SW480 cells and T47D cells transfected with the construct P1 representing 1858 bp of the promoter showed a 2- to 3-fold increase of luciferase activity after treatment with TPA and A23187 in comparison with unstimulated cells. Comparable levels of induction were found for P2. Lower levels of luciferase activity could be seen for the construct P3 (Figure 6B). We could see that the promoter region of −377 to −624 bp could be important for the CEACAM1 expression after treatment with TPA and ionophore not only for the endometrial carcinoma but also for carcinoma cell lines of the breast and colon cancers.

Discussion

In this study, we tested the effect of different hormones and cytokines on the induction of expression in the CEACAM1 negative endometrial adenocarcinoma cell line Hec1B. No induction of mRNA expression was observed after treatment with estradiol, MPA or RU486 in parental Hec1B cells and after transfection of the corresponding receptors. Furthermore, no effect on the CEACAM1 expression was found after stimulation with IFN-γ and TNF-α demonstrating that both cytokines and the hormones estradiol and MPA are not involved in the regulation of CEACAM1 in Hec1B cells. In contrast, treatment of Hec1B cells with the phorbol ester TPA and the calcium ionophore A23187 resulted in the strong induction of CEACAM1 mRNA and protein expression. TPA is a strong activator of protein kinase C (PKC) and increased cytoplasmic PKC activity and inhibition of proliferation in a dose-dependent manner has previously been reported for

**Effect of actinomycin D and cycloheximide on the induction of CEACAM1 expression**

Hec1B cells were treated with TPA and ionophore A23187 in the absence and presence of actinomycin D and cycloheximide. Levels of mRNA expression were investigated by northern blot analysis (Figure 5). In contrast to the strong induction of expression after treatment with TPA and ionophore A23187, no CEACAM1 transcripts were detectable in the presence of actinomycin D or cycloheximide indicating that the re-expression of CEACAM1 is dependent on the synthesis of mRNA and protein and possibly activated at the transcriptional level.

**Fig. 5.** (A) Effect of actinomycin D (ActD) and cycloheximide (Chx) on the induction of CEACAM1 mRNA expression after treatment with TPA and calcium ionophore of Hec1B cells. (B) Rehybridization of the membrane with G3PDH. No effect of actinomycin D or cycloheximide on the expression of CEACAM1 was observed in the absence of TPA and calcium ionophore; no induction was observed in response to DMSO used as a solvent (data not shown).
Hec1B cells in response to TPA (43). Calcium ionophore acting via the increase of cytosolic calcium levels is also known to activate PKC (44) explaining the additive effect of both substances on the induction of CEACAM1 in Hec1B cells. In contrast to Hec1B cells, no induction of CEACAM1 expression was observed in endometrial mixed mesenchymal Skut1B cells. The stimulation of the PKC isoenzyme system is a common step in many signal transduction pathways, and it is well known that the activation of PKC by TPA results in diverse effects in different cells (42). The different response to TPA and calcium ionophore A23187 observed in HEC1B and Skut1B cells is probably due to cell type-specific differences in the expression pattern of PKC isoforms and the activation of subsequent signaling pathways. In a study, which we have already published, we studied the functional role of differential PKC isoform expression in uterine tumor progression and have compared the proliferative response to TPA, changes in cell morphology induced by TPA, and the PKC isoform expression pattern in HEC1B and Skut1B cells. The moderately differentiated endometrial HEC1B cell line showed a marked increase in proliferative activity and a profound morphological change in response to TPA, but Skut1B cells did not. Analysis of the PKC isoform expression profile by western blot revealed that PKC α, βI, δ, ε and ζ were expressed at a much higher level in HEC1B as compared with Skut1B cells. PKC βII was the only isoenzyme to exhibit a higher expression level in Skut1B cells. These data demonstrate that the response to TPA correlates with the expression levels of the majority of PKC isoforms in these cells (45).

Fournier et al. (46) investigated the PKC isozyme profile of...
endometrial carcinomas from 42 patients who were not previously exposed to antiestrogens by western blot. They could demonstrate that PKCα and ER expression are inversely related in endometrial cancer. Because of the parallel induction of CEACAM family members previously observed in response to cytokines (33) and the relatively high homology of the upstream regulatory regions of the family members (36), we investigated the effect of TPA and ionophore A23187 on the expression of CEACAM5, CEACAM6 and CEACAM7, respectively. As demonstrated by northern and western blot analysis, induction of expression by TPA and ionophore A23187 was restricted to CEACAM1, whereas no effect on the mRNA and protein expression was observed for the other members of the CEACAM family. Parallel and differential expression of individual members of the CEACAM family have been reported in cell lines and different human tissues (1,2). Restricted expression of CEACAM1 was observed e.g. in the human endometrium and in the prostate while co-expression of several members of the CEACAM family was found in several organs of the gastrointestinal tract (5,7,8,20). These observations together with the findings in the present study indicate that the expression of members of the CEACAM family can be regulated independently—possibly due to the expression of tissue-specific transcription factors.

For a better understanding of the regulatory mechanisms involved in the induction of CEACAM1 in Hec1B cells, expression studies were performed in the presence of the transcriptional and translational inhibitors actinomycin D and cycloheximide, respectively. The induction of CEACAM1 expression was completely abrogated indicating that the re-expression of CEACAM1 is dependent on the transcription and synthesis of regulatory factors induced by PKC in turn leading to the activation of CEACAM1 (45). Comparison with corresponding consensus sequences of transcription factors reveals that several potential binding sites are present in the human CEACAM1 promoter. Chen et al. (41) showed that CEACAM1 promoter contains an interferon-sensitive response element (ISRE). Interferon regulatory factor-1 (IRF-1) was identified as the ISRE-binding factor. A second analysis revealed the binding of SP1, an SP1-like protein, and upstream stimulatory factor (24). The SP1-like complex was also induced by IFN-γ treatment of the colorectal HT29 cells (24). To further determine the functional significance of these putative binding sites, luciferase assays with Hec1B cells were performed and confirmed that the induction of CEACAM1 is transcriptionally regulated involving a region of 247 bp localized 377 bp upstream of the start codon. Further studies with SW480 and T47D cells showed similar results, but luciferase assays demonstrated that the transcriptional activity of these cell lines after stimulation with TPA and ionophore A23187 was lower in comparison with HEC1B cells (Figure 6A and B). Scanning this region by computer programs identified AP1, AP2, NFκB, IRF-1 and SP1-like complex as putative transcription factors, binding in this region and which could be activated through TPA and A23187. We conclude, that activation of this region of the CEACAM1 promoter could be relevant for the CEACAM1 expression not only in endometrial carcinoma cells but also for breast and colon carcinoma cells. Our results show, that the increased expression of CEACAM1 by TPA and ionophore A23187 seems to be physiologically relevant and not only related to a specific property of the endometrial carcinoma cell line Hec1B.

To our knowledge this is the first report demonstrating that the expression of CEACAM1 is inducible by phorbol ester and calcium ionophore. It is well documented that both substances are acting via the activation of PKC, indicating that PKC plays an important role in the regulation of CEACAM1. In this context it is interesting to note that decreased levels of PKC expression and activity were observed in colorectal adenomas and carcinomas in comparison with normal mucosa (47,48). Moreover, colonic tumor growth was suppressed when isoforms of PKC were overexpressed and a reduction in invasiveness of bladder carcinoma cells was reported after PKC stimulation (49,50). However, previous studies and the findings in this study link the decrease of PKC activity to the loss of CEACAM1 expression in carcinomas of the endometrium, colon and breast and may therefore have important clinical implications. As CEACAM1 is acting as a tumor suppressor and is known to be downregulated in several human tumors, restoration of the expression of CEACAM1 e.g. by non-toxic analogues of TPA or calcium ionophore A23187 may inhibit the growth of certain tumors.

Acknowledgements

The cosmid clone R26908 for the generation of promoter constructs was a generous gift of Dr A.S.Olsen to whom we are very thankful (Lawrence Livermore National Laboratories, CA). DNA of expression vectors coding for the estrogen, progesterone A and progesterone B receptor were a kind gift of P. Chambon, Strassbourg, France). The authors would like to thank Mr J. Koppelmeyer for help with photographic work.

Conflict of Interest Statement: None declared.

References


Received July 14, 2005; revised November 11, 2005; accepted November 12, 2005