

Telomerase activity in gestational trophoblastic disease and placental tissue from early and late human pregnancies

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BACKGROUND: The aim of this study was to evaluate telomerase activity in tissue from cases of gestational trophoblastic disease (GTD) and in placental tissue from early and late human pregnancies. **METHODS:** We used a telomeric repeat amplification protocol assay to measure telomerase activity in 132 tissue samples from normal early pregnancies, spontaneous abortions, normal late pregnancies, cases of late-pregnancy intrauterine fetal death, and GTD. **RESULTS:** Telomerase activity was detected more often in normal early pregnancies and cases of GTD than in spontaneous abortions and normal late pregnancies ($P < 0.001$). During early gestation, no significant difference in detection rates was found between normal pregnancies and complete hydatidiform mole. As gestational age increased, detection rates for normal pregnancies decreased significantly ($P = 0.0001$), while for complete hydatidiform mole no significant changes occurred. **CONCLUSIONS:** Our findings indicate that placental tissue from normal early pregnancies and neoplastic tissue from GTD possess similar levels of telomerase activity. Decreasing regulation of telomerase activity is present in normal pregnancies but not in complete hydatidiform mole. The fact that telomerase activity decreases in cases of fetal demise, and as pregnancy progresses, also suggests that placental senescence may play a role in the development and ageing of the placenta.

Key words: abortion/complete hydatidiform mole/pregnancy/telomerase activity

Introduction

Telomerase is a cellular reverse transcriptase that adds 5'-d(TTAGGG)-3' hexameric repeats onto the 3' ends of chromosomes, thus helping to provide genomic stability by maintaining the integrity of these chromosome ends. Telomerase activity is associated with the majority of malignant human cancers. In cancer cells, augmentation of telomerase activity appears to be necessary to balance telomere loss, thus maintaining a telomere length sufficient to ensure proliferation (Counter *et al.*, 1994; Collins *et al.*, 1995). Normal human somatic cells have a limited life span both *in vitro* and *in vivo* and are mostly telomerase-negative. It has been suggested that this limited capacity for proliferation is also regulated by telomere length (Harley, 1991; Allsopp *et al.*, 1992).

Gestational trophoblastic disease (GTD) refers to a category of neoplasm that includes complete hydatidiform mole, partial hydatidiform mole and choriocarcinoma (World Health Organization, 1983; Kohorn *et al.*, 2000). All of these tumours are pregnancy-related and possess a proliferative neoplastic trophoblast. In normal pregnancies, a villous trophoblast in the placenta consists of a population of proli-

ferating cytotrophoblasts that differentiate and individually fuse into a syncytiotrophoblast (Huppertz *et al.*, 1998). In the first trimester, the cytotrophoblast proliferation rate is high (Castellucci *et al.*, 2000). The proportion of proliferative villous cytotrophoblasts among the total trophoblast population decreases after the first trimester. In the third trimester, the overall proliferation rate for the trophoblasts falls to nearly 10% of the first trimester value (Castellucci and Kaufmann, 2000). In spontaneous abortions, the trophoblasts are less hyperplastic (van Lijnschoten *et al.*, 1994) and degenerative lesions are frequently found in the placental tissue (Larsen and Graem, 1999; Benirschke and Kaufmann, 2000). Therefore, trophoblasts from the placentas of normal pregnancies, abortions, and cases of intrauterine death, and from tissue samples from cases of GTD, seem to differ in terms of their respective rates of proliferation and degeneration. At present, no study has specifically compared these several kinds of tissue with regards to the presence of telomerase activity.

The aim of our study was to evaluate and compare the occurrence of telomerase activity in tissue samples from normal pregnancies, spontaneous abortions, cases of intrauterine fetal death (IUFD) and cases of GTD.

Materials and methods

Tissue samples

A total of 132 tissue samples containing trophoblasts were included in the study. The samples came from patients with GTD and women who either underwent an abortion early in pregnancy or gave birth during late pregnancy: 21 came from women with GTD (18 complete hydatidiform mole, three choriocarcinoma), 45 from women who underwent a legal abortion for social reasons during early normal pregnancy (5–12 weeks), 37 from women who gave birth during a late normal pregnancy (35–42 weeks), 27 from women who had a surgical evacuation because of a spontaneous abortion (6–13 weeks), and two from women who experienced late-pregnancy IUD. The Ethics Committee of National Taiwan University Hospital approved the study, and all patients gave informed consent.

We began by washing these tissue samples in an ice-cold wash buffer [10 mmol/l Hepes-KOH (pH 7.5), 1.5 mmol/l MgCl₂, 1 mmol/l EGTA, 10 mmol/l KCl, 1 mmol/l dithiothreitol] and then used liquid nitrogen to shock freeze the samples into smaller pieces. Afterwards, we used disposable surgical knife blades to slice flakes from these frozen tissue specimens, which had previously been prepared on sterile Petri dishes; these flakes were immediately transferred to homogenization tubes containing 200 µl ice-cold lysis buffer [10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l MgCl₂, 1 mmol/l EGTA, 0.1 mmol/l phenylmethylsulphonyl fluoride, 5 mmol/l β-mercaptoethanol, 0.5% CHAPS (Sigma, St Louis, MO, USA) and 10% glycerol]. The flakes were then homogenized on ice with a motorized pestle until they reached a uniform consistency. After incubating the lysate on ice for 30 min, it was centrifuged at 16 000 g for 20 min at 4°C using Eppendorf tubes. We then carefully removed the supernatant and measured the protein concentration by Bradford assay (Bradford, 1976). Finally, we used liquid nitrogen to shock freeze the tissue extracts into aliquot parts, and then stored them at –80°C.

Telomerase repeat amplification protocol (TRAP) reaction

We performed a telomerase repeat amplification protocol (TRAP) assay using the Telomerase PCR ELISA assay in a 50 µl reaction mixture according to the manufacturer's protocol (Boehringer Mannheim Biochemicals, Mannheim, Germany). In this primer-extension based assay for detecting telomerase activity, the telomerase reaction product is amplified by PCR (Kim *et al.*, 1994) and a photometric enzyme immunoassay is used. We began by mixing a 25 µl reaction solution—which contained a Tris buffer, a biotin-labelled P1-TS primer, a P2 primer, nucleotides and a Tag polymerase—with 3 µl of cell extract. We then added sterile water to the result, until a final volume of 50 µl was reached. After a 30 min incubation period at 25°C for telomerase-mediated extension of the P1-TS primer, the reaction mixture was heated to 94°C for 5 min and immediately subjected to 33 PCR cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. After adding 5 µl of the amplification product and 20 µl of a denaturation reagent containing sodium hydroxide (<0.5%) to 225 µl of hybridization buffer (digoxigenin-labelled detection probe), we mixed the resulting combination thoroughly.

Hybridization and ELISA procedure

We then transferred 100 µl of the mixture to a well made from a streptavidin-coated microtitre plate. We incubated the microtitre plate at 37°C on a shaker for 2 h, and then washed it three times with 250 µl of washing buffer. After adding 100 µl of anti-digoxigenin-peroxidase and incubating at room temperature for 30 min, while shaking, we removed the solution. We then added 100 µl of a substrate solution containing 3,3',5,5'-tetramethyl benzidine and

incubated at room temperature for 10–20 min for colour development while still shaking. Finally, we added 100 µl of 5% sulphuric acid to stop the reaction. Using a microtitre reader, we measured the absorbance of the samples at 450 nm. Absorbance values were reported as the A_{450 nm} reading against blank (reference wavelength A_{690 nm}). We regarded samples as telomerase-positive if the difference in absorbance (ΔA) was higher than 0.25 units. For negative controls, we incubated 5 µl of cell extract with DNase-free RNase at a concentration of 1 µg/µl for 20 min at 37°C. The maximum value of absorbance for the negative control should be 0.25 units; if the value was higher, the whole test, including TRAP, was repeated. A cell extract prepared from immortalized telomerase-expressing human kidney cells was used as a positive control. Since the absorbance readings of the positive controls should be >1.5 units, the test was repeated if the values were lower.

Results

A total of 132 patients supplied the tissue samples that were used in this study. Clinical characteristics such as age, parity and week of gestation (for normal pregnancies, spontaneous abortion and complete hydatidiform mole) are shown in Table I. Although there were no significant differences in age (one-way analysis of variances), patient's parity before their current pregnancy differed significantly ($P = 0.0003$, Pearson's χ^2 test). Multiparity was found more often in women who had requested a legal abortion during early pregnancy. The mean fetal birth weight for the women who gave birth during a late normal pregnancy was 3334 g (SD 301, range 2620–3882). Seventeen of these babies were male and 20 were female; neither they nor their mothers experienced any problems in the post-natal period. Nothing unusual was discovered after placentas from cases of normal early pregnancy and normal late pregnancy were checked for their gross appearance. Further, all tissue specimens from cases of spontaneous abortion were submitted for pathological examination; microscopic analysis disclosed a histological picture marked by decidua, trophoblasts, oedematous chorionic villi, haemorrhaging, adjacent decidualized endometria and increased perivillous fibrin deposition. Chorionic villi were also found to have focally degenerative cysternal changes.

Both patients who experienced intrauterine fetal death were nulliparous. One was a 36-year-old who had three seizures during week 37 of pregnancy. After reaching a diagnosis of eclampsia and intrauterine fetal death, a Caesarean section was performed that delivered a macerated male baby, 2576 g in weight. The other patient was a 30-year-old woman who, after not experiencing a fetal movement for 1 day, sought help at our hospital. After determining that intrauterine death had occurred, labour was induced and a dead 2550 g macerated male baby, with dark yellowish and diffuse meconium staining in the amniotic fluid, was delivered. Marked fibrotic change was found in both placentas, and marked calcification and multiple infarcts were also found in the case of eclampsia.

For patients with GTD, an initial work-up was carried out which included chest roentgenography and liver and renal function tests. Patient data included age, gravidity, parity, uterine size at the time of evacuation, method of termination, pre-evacuation serum β-HCG level, radiological findings, histo-

Table I. Clinical characteristics

| | Normal late pregnancy (n = 37) | Normal early pregnancy (n = 45) | Spontaneous abortion (n = 27) | Complete hydatidiform mole (n = 18) | P |
|-----------------|-----------------------------------|------------------------------------|----------------------------------|--|---------------------|
| Age (years) | | | | | NS ^a |
| Mean ± SD | 31.5 ± 3.8 | 31.1 ± 5.5 | 29.8 ± 4.5 | 33.5 ± 5.4 | |
| Range | 26–43 | 19–41 | 24–39 | 22–48 | |
| Previous births | | | | | 0.0003 ^b |
| 0 | 16 (43) | 7 (16) | 8 (30) | 7 (39) | |
| 1 | 17 (46) | 16 (35) | 17 (63) | 7 (39) | |
| 2 | 4 (11) | 22 (49) | 2 (7) | 4 (22) | |
| Gestation weeks | | | | | NS ^b |
| 5–10 | – | 31 (69) | 18 (67) | 13 (72) | |
| 11–13 | – | 14 (31) | 9 (33) | 5 (28) | |

^aDetermined by one-way analysis of variances.

^bDetermined by Pearson's ² (exact) test.

Percentages are shown in parenthesis.

NS = not significant.

logic evaluation and laboratory findings. Seventeen of the 18 patients with complete hydatidiform mole were treated with suction curettage. The resulting evacuated specimens were all found to have translucent molar vesicles. Microscopic analysis showed marked hydropic change and oedema and cysterna formation on the villi, a villous core that lacked fetal capillaries, and marked hyperplastic trophoblasts on the villous surface. The patients were tested weekly after evacuation until three consecutive negative serum β -HCG levels were obtained, and afterwards were tested at 1 month intervals for at least 1 year. Patients with a β -HCG plateau that lasted >3 weeks, who had an elevated level for >2 weeks, or who had any sign of metastasis were diagnosed as having persistent GTD. Among the 18 cases of complete hydatidiform mole, two developed persistent GTD and 16 spontaneously regressed. Both of the patients with persistent GTD underwent chemotherapy and achieved remission: one underwent single agent chemotherapy with methotrexate for three courses and was then shifted to a combination chemotherapy with EMACO (etoposide, actinomycin-D, methotrexate, vincristine and cyclophosphamide), while the other underwent seven courses of single agent chemotherapy.

Of the three patients with choriocarcinoma, two underwent an initial surgical operation before chemotherapy and one underwent surgery during the course of chemotherapy. In one case, that of a nulliparous woman, there was an initial clinical impression of ectopic pregnancy and myoma uteri. During her operation, a 7×5×5 cm myoma was found on the uterine fundus, while on the myoma there was a 5×4.5×4 cm haemorrhagic tumour mass. Local excision of the myoma together with the haemorrhagic tumour was performed. The other patient underwent an emergency laparotomy because of heavy intra-abdominal bleeding. During her operation, a subtotal hysterectomy was performed after a 6×5.5×5 cm actively bleeding mass was discovered on the uterine fundus. Microscopically, the haemorrhagic tumours from both cases showed a choriocarcinoma composed of clusters of cytotrophoblasts that were separated by streaming syncytiotrophoblast masses. Evident haemorrhage and necrosis were also noted,

Table II. Expression of telomerase activity in tissue from cases of gestational trophoblastic disease and in placental tissue from early and late human pregnancies

| | Positive | Negative | Total |
|---|----------|----------|-------|
| Normal early pregnancy | 33 (73) | 12 (27) | 45 |
| Spontaneous abortion | 9 (33) | 18 (67) | 27 |
| Normal late pregnancy | 11 (30) | 26 (70) | 37 |
| Late-pregnancy intrauterine fetal death | 0 (0) | 2 (100) | 2 |
| Complete hydatidiform mole | 15 (83) | 3 (17) | 18 |
| Choriocarcinoma | 3 (100) | 0 (0) | 3 |

Percentages are shown in parenthesis.

but no chorionic villus was seen. In both cases, remission was achieved after post-operative chemotherapy with EMACO. The third patient had a choriocarcinoma that had metastasized to her lungs, intestines and brain. This patient underwent seven courses of chemotherapy with EMACO and two courses with Ifosfamide and cisplatin. During the treatment period, severe intestinal bleeding due to jejunum metastasis occurred. A segmental resection of the intestine was performed for treatment of the intestinal bleeding. Histopathological examination revealed a picture of metastatic choriocarcinoma. Irradiation therapy to the whole brain for 3000 cGy was also performed for the brain metastasis. Unfortunately, she eventually died.

Telomerase activity in cases of normal pregnancy, spontaneous abortion, IUFD and GTD is shown in Table II. Fifteen of the 18 (83%) cases of complete hydatidiform mole and all three cases of choriocarcinoma tested positive for telomerase activity. Of the 45 placental tissue samples from early pregnancies that we examined using the TRAP assay, 33 (73%) tested positive for telomerase. In contrast, only 11 of the 37 (30%) late pregnancy samples expressed telomerase activity. Samples from early spontaneous abortions also exhibited telomerase activity, but at a low level; only nine of the 27 (33%) tested positive for telomerase activity. Telomerase activity was not detected in the placental tissue from the two cases of late-pregnancy intrauterine fetal death.

Table III. Positive rates of telomerase activity in placental tissue from normal early pregnancies and in tissue samples from cases of complete hydatidiform mole for gestation periods of <10 weeks and ≥10 weeks

| Gestation period pregnancy | Normal early abortion | Spontaneous mole | Complete hydatidiform | <i>P</i> ^a |
|----------------------------|-----------------------|------------------|-----------------------|-----------------------|
| <10 weeks | 25/31 (81) | 7/18 (39) | 10/13 (77) | 0.0077 |
| ≥10 weeks | 8/14 (57) | 2/9 (22) | 5/5 (100) | 0.0187 |

^aPearson's χ^2 (exact) test. Percentages are shown in parenthesis.

We observed significant telomerase activity in the placental tissue that came from early stage pregnancies. A comparison of different pregnancies for positive telomerase activity in the early gestation period between <10 weeks and ≥10 weeks is shown in Table III. Tissue samples from the placentas of normal pregnancies, spontaneous abortions and cases of GTD showed different rates of telomerase activity. In the early normal pregnancies before 10 weeks, telomerase activity was detected in the majority of the placental tissue samples from early pregnancies (81%) and in most of the tissue samples with neoplastic trophoblasts from cases of complete hydatidiform mole (77%), while few of the placental tissue samples from spontaneous abortions displayed telomerase activity (39%). Thus, there was no significant difference in the presence of telomerase activity between tissue samples from normal early placentas and from samples with neoplastic trophoblasts (Fisher's exact test), with both normal early placental tissue and neoplastic trophoblastic tissue having higher positive rates of telomerase activity than placental tissue from spontaneous abortions (*P* = 0.0036 and *P* = 0.0343 respectively). For early pregnancies, at or after 10 weeks, although telomerase activity was present more often among tissue samples with neoplastic trophoblasts (100%) than among placental tissue from spontaneous abortions (22%, *P* = 0.0210), no significant difference was found to exist between placentas from early normal pregnancies (57%) and those from spontaneous abortions. There was also no significant difference in the presence of telomerase activity between normal pregnancies and molar pregnancies (Fisher's exact test).

In normal pregnancies without symptoms of abortion, telomerase activity was present less frequently as the pregnancy progressed. It was found in 81% (25/31) of the pregnancies that were tested before 10 weeks, 57% (8/14) of the early pregnancies after 10 weeks, and 30% (11/37) of the late pregnancies (*P* = 0.0001, Figure 1). In cases of complete hydatidiform mole, however, this phenomenon of decreasing activity was not present (10/13, 77% versus 5/5, 100%, *P* = 0.3505); nor was it found in spontaneous abortions (7/18, 39% versus 2/9, 22%) or late pregnancy IUID (0/2, Figure 1).

Among the 16 cases of complete hydatidiform mole that resulted in spontaneous remission after evacuation, 13 tested positive for telomerase activity and three tested negative. The two cases of complete hydatidiform mole that subsequently developed persistent trophoblastic disease both tested positive for telomerase activity. Thus, of the 15 patients with complete hydatidiform mole that tested positive for telomerase activity,

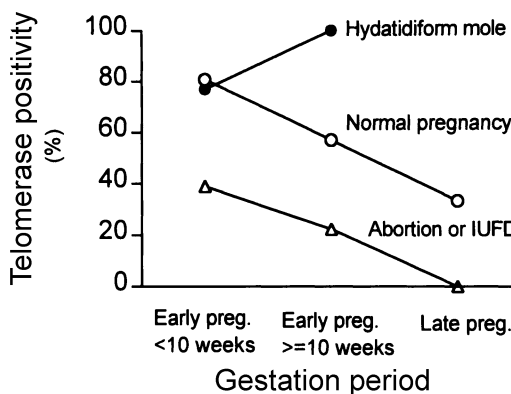


Figure 1. Positive rates of telomerase activity in tissue samples from cases of complete hydatidiform mole and from the placentas of normal pregnancies, abortions and cases of intrauterine fetal death.

only two later developed persistent GTD. However, none of the three patients with negative telomerase activity later developed persistent GTD.

Discussion

During early pregnancy, some trophoblasts penetrate the endometrium and proliferate, thus causing the placenta to develop rapidly (Lopata, 1996). This ability to rapidly proliferate, coupled with a high capacity for invasion (Bamberger *et al.*, 1999; Bischof and Campana, 2000; Hemberger *et al.*, 2000) can confer on placental tissue a tumour-like character (Khoo *et al.*, 1998). It was this proliferative and invasive nature of the trophoblast that prompted us to examine telomerase activity in the tissue from normal pregnancies and cases of GTD.

In our study, we found that telomerase activity was at its highest during the early period of normal pregnancy. We also found that this activity decreases significantly after the first trimester. Our findings suggest that in normal pregnancies, telomerase was down-regulated as the pregnancy progressed. However, the mechanism that underlies this phenomenon is not known. As for placental tissue from normal pregnancies, although there is a subpopulation of trophoblasts (cytotrophoblasts) that may be proliferative, other cytotrophoblasts and the syncytiotrophoblast are not proliferative. The proportion of proliferative villous cytotrophoblasts among the total trophoblast population decreases as the pregnancy progresses from the first trimester to term (Castellucci and Kaufmann, 2000). Whether isolated proliferating cytotrophoblast cells possess

a higher level of telomerase activity than non-proliferating cytotrophoblast cells or the syncytiotrophoblast needs further study.

Maintenance of telomerase activity has been associated with increased cellular resistance to apoptosis (Holt *et al.*, 1999). Apoptosis (programmed cell death) leads to the elimination of old, unnecessary and unhealthy cells. In recent studies of first trimester placental tissue by means of either haematoxylin or eosin staining (Smith *et al.*, 1997) or by electron microscopic assessment (Smith *et al.*, 2000), apoptosis was discovered in the human placenta. These studies also found that the incidence of placental apoptosis increased with increasing gestational age.

To our knowledge, there have been no reports of telomerase activity in cases of spontaneous abortion or IUDF. In this study, we found that rates of positive telomerase activity are low for cases of fetal death no matter when they occurred. Telomerase activity was rarely present in cases of spontaneous abortion, and not present at all in cases of fetal demise. In recent reports that evaluated telomerase activity in the placenta in cases with or without fetal growth retardation, telomerase activity was detected more often in cases without fetal growth retardation (Isuzu *et al.*, 1999; Kudo *et al.*, 2000). Furthermore, increased apoptosis has also been found in the villi from spontaneous abortions (Qumsiyeh *et al.*, 2000). From both these reports and our data, we hypothesize that the down-regulation of telomerase activity in normal pregnancies may play a role in placental senescence and may be connected to placental apoptosis.

Complete hydatidiform mole is an abnormal conceptus. Whether neoplastic trophoblasts from molar tissue samples and normally proliferative trophoblasts from early pregnancy placental tissue samples possess the same degree of telomerase activity has rarely been investigated. In our study, we detected telomerase activity in the majority of the tissue samples from both normal early pregnancies and from cases of complete hydatidiform mole. In fact, during the early stages of gestation, there was no significant difference between them. This finding suggests that placental tissue that has a tumour-like character during early pregnancy and neoplastic trophoblastic tissue both possess the same proliferative ability.

In addition to these findings, we discovered that in cases of complete hydatidiform mole, telomerase activity remained constant as the pregnancy progressed. This contrasts with what we found in normal pregnancies, where telomerase activity was present less often in the later stages of pregnancy, and indicates that the down-regulation of telomerase activity that occurs in normal pregnancies is not present in complete hydatidiform mole. These results support the hypothesis that complete hydatidiform mole is essentially a neoplasm, and that telomerase activity provides a mechanism for avoiding the proliferative limitation due to telomere loss.

Furthermore, in our study three cases of choriocarcinoma tested positive for telomerase activity. To our knowledge, in previous studies only six cases of choriocarcinoma had been tested for the presence of telomerase; all tested positive for telomerase activity (Bae and Kim, 1999; Cheung *et al.*, 1999). The high rates of telomerase activity for choriocarcinoma suggest that this tumour is highly oncogenic, and that such

activity may be a critical step in the oncogenesis of this malignancy.

In our study, we found that telomerase activity occurs similarly in both placental tissue from normal early pregnancies and neoplastic trophoblastic tissue. Thus, our findings suggest that proliferative normal trophoblasts and neoplastic trophoblasts possess similar levels of telomerase activity. In normal pregnancies, telomerase activity is down-regulated over the course of gestation, while in cases of hydatidiform mole it is not. The fact that telomerase activity decreases in cases of fetal demise, and as pregnancy progresses, also suggests that placental senescence may play a role in the development and ageing of the placenta, but not in the appearance and growth of trophoblasts in cases of complete hydatidiform mole.

Acknowledgements

This work was supported in part by Research Grants NSC88-2314-B-002-386 and NSC89-2314-B-002-206 from the National Science Council.

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Submitted on April 17, 2001; resubmitted on August 13, 2001; accepted on October 15, 2001