Increased autophagy in the placental territory of selective intrauterine growth-restricted monochorionic twins

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ABSTRACT

Subject This study investigates the placental autophagic activity in growth-restricted fetuses in the monochorionic (MC) twin model.

Patients and methods Forty MC twins were prospectively enrolled in this study, including 21 with and 19 without selective intrauterine growth restriction (sIUGR), defined as birth weight below the tenth percentile. The sIUGR group was subdivided on the basis of present versus absent or reversed umbilical artery end-diastolic flow at Doppler. Placenta samples were taken after delivery from each twin placenta territories. After protein extraction, Western blot was used to determine light chain 3 (LC3)-II placental protein expression in sIUGR and appropriately grown (appropriate-for-gestational age, AGA) twins.

Results The LC3-II was significantly higher in the sIUGR twin placental territory than in their AGA counterparts. In the sIUGR group, LC3-II fold change was significantly increased compared with that in the AGA cotwins (2.28 vs 1.04, \(p<0.05\)). Placental LC3-II protein expression was particularly stimulated in the MC sIUGR group with abnormal umbilical artery Doppler flow compared with AGA controls (\(p<0.05\), one-way analysis of variance test).

Conclusions In MC twins, the placental autophagic activity is different between sIUGR and AGA cotwins. The placenta territory with the least blood flow perfusion has the highest autophagic activity. © 2013 John Wiley & Sons, Ltd.

INTRODUCTION

Autophagy, also called type II programmed cell death, is a catabolic process used by eukaryotic cells to recycle long-lived proteins and lipids and eliminate protein aggregates and organelles. Autophagic activity is upregulated by starvation, growth factor deprivation, and hypoxia. In monochorionic twin (MC) pregnancies with selective intrauterine growth restriction (sIUGR), the umbilical blood flow perfusion to the sIUGR twin is significantly decreased versus that to the appropriate-for-gestational age (AGA) cotwin. This poor perfusion condition is similar to starvation, growth factor deprivation, or hypoxia. Microtubule-associated protein 1A/1B light chain 3 (LC3) is a soluble protein located in mammalian cells. During autophagy, autophagosomes engulf intracellular proteins and organelles. At the same time, the cytosolic form of LC3, also called LC3-I, is linked to phosphatidylethanolamine. This conjugated form of LC3 is LC3-II. LC3-II attaches to the membrane of autophagosome, which then fuses with lysosome to form autolysosome. During the process of autophagy, intra-autophagosomal components are degraded by lysosomal hydrolases. At the same time, LC3-II is also degraded. Thus, LC3-II can reflect autophagic activity and autophagy-related process.

The role of autophagy in IUGR has been reported. Hung et al. demonstrated increased autophagic activity in IUGR placentas of singleton pregnancies. However, the study design to evaluating IUGR fetuses faced a difficult problem, namely that the comparison between the IUGR group and the normal control group was mainly based on fetal weight difference and did not take into consideration the intragroup heterogeneity. In the MC setting, when comparison is drawn between the twins, the need to adjust for confounding factors is minimized to a great extent. MC is one type of monozygotic twins in which both fetuses share the identical genomic DNA and grow under the same maternal environment. It is thus the most ideal environment to evaluate the role of autophagic activity in IUGR.

The IUGR in MC twins frequently involves only one of the fetuses, hence the name of sIUGR. sIUGR occurs in about 12% of twin pregnancies, and those fetuses are at high risk of neurological damage. In MC twins with sIUGR, the AGA cotwin shows genetically determined growth potential; in contrast, the sIUGR fetus presents the failure to achieve its genetically determined growth potential. The AGA fetus of MC twins can be
used as a control of the sIUGR cotwin, eliminating the bias of genetic, environment, gestational age, and maternal condition.

The placenta of MC twins can be separated into two placental territories using the vascular equator as boundary. Usually, the portion of sIUGR placenta territory is smaller and with less umbilical flow perfusion than that of the AGA cotwin.8 We assumed that different perfusions in the MC twins will be associated with different autophagic activities. Thus, the purpose of this study is to investigate the effect of poor perfusion in the sIUGR fetus on the placenta autophagic activity using an MC twin model.

MATERIAL AND METHODS

We collected prospectively the placentas from women who delivered live MC twins at the Chang Gung Memorial hospital and whose placentas were intact enough to be studied. All MC twins were delivered by Cesarean section. MC twin pregnancies were diagnosed prenatally by ultrasound as having a single placenta, having a thin dividing membrane, and lacking a twin peak sign. An MC twin pregnancy with sIUGR was defined as an estimated fetal weight below the tenth percentile in one twin.10 Placentas from MC twin pregnancies without twin–twin transfusion syndrome or sIUGR delivered during the same period were used as controls. The diagnosis of twin–twin transfusion syndrome was based on the ultrasound findings defined by Quintero et al.11 In MC twins with sIUGR, the sIUGR twin was the twin with birth weight below the tenth percentile, and the AGA twin was the cotwin without IUGR. Umbilical artery (UA) Doppler was normally done as abnormal if it has absent or reversed end-diastolic flow. Twins with anomalies or one fetal death were all excluded from the study. Informed consents were obtained from each patient before delivery. This study was approved by the local institutional ethics committee.

Placenta collection

Two or three pieces of placenta (0.5 × 0.5 × 0.5 cm) from two individual placenta territories were collected fresh by cutting about midway between the vascular equator and the individual cord insertion and at the middle layer of placenta between the maternal and fetal surfaces. The vascular equator was defined as a border drawn in the middle of the avascular zone on the chorionic fetal surface when there were no intertwin vascular anastomoses or on the anastomosis points where twin–twin communicating vessels met.8 Areas including calcification and infarction were avoided. The placenta specimens were briefly rinsed with ice-cold phosphate-buffered saline to clear blood. Then, the placental tissue was frozen in liquid nitrogen and stored at −70 °C condition. After the placenta is sampled for protein extraction, it was freshly cut along the vascular equator. The placentas were cut along the line that divided the placenta into two territories between both cord insertions. Each placental portion was weighed separately, which yielded an estimated individual placental mass. The placental territory was calculated as the estimated placenta weight of one twin/total placenta weight × 100%. The presence of velamentous cord insertion (VCI) was documented.

Western blots analysis

Proteins were extracted using radioimmunoprecipitation assay buffer (50 mmol/L Tris–Cl, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, pH 7.5) containing 1 mmol/L phenylmethylsulfonyl fluoride and quantified with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). One hundred micrograms of placental proteins was separated by 12% sodium dodecyl sulfate polyacrylamide electrophoresis and then transferred to a nitrocellulose membrane. These proteins were probed with primary antibodies against human LC3-1 and LC3-II (1:2000, Novus Biologicals, Littleton, CO) at 4 °C overnight. The membrane was washed by Tris-buffered saline and Tween 20 for three times and then probed with secondary antibody for 2 h. The proteins were stained by enhanced chemiluminescence (Millipore WBKLS0500, PIERCE prod.#32209 or Santa Cruz sc-2049), and the LC3-II protein content was normalized by β-actin as fold change.

The LC3-II fold change ratio (FCR) in a twin pair was defined as LC3-II protein fold change of the smaller (sIUGR) twin/LC3-II protein fold change of the larger (AGA) twin, which represented autophagy activity of the smaller (sIUGR) twin’s placenta territory using autophagic activity of the larger (AGA) twin’s placenta territory as internal control.

Birth weight discordance was calculated as the difference between the birth weights of the larger twin and the smaller twin divided by the weight of the larger twin. The placental territory discordance was also calculated in a similar manner.

Statistical analysis

Statistical analysis was conducted with SPSS software (version 11.0 for Window; SPSS Inc, Chicago, IL). The p-value of protein expression fold change was calculated by paired t-test. Two-sample Student’s t-test or Mann–Whitney U-test was used for comparisons of continuous variables. An analysis of variance test was applied to compare the values among the AGA MC twins and those with normal or abnormal UA Doppler. A p-value <0.05 was considered statistically significant.

RESULTS

Clinical characteristic

In this study, 40 pairs of MC twins were enrolled prospectively: In 21, one twin had sIUGR, and in 19, both twins were AGA. Between the two groups, there was no significant difference in maternal age, but the MC twin pregnancies with sIUGR were delivered significantly earlier, and the birth weights were lighter than those of MC twin pregnancies without sIUGR. VCI was more frequent in the sIUGR twin placentas (Table 1).

Placental LC3-II expression

Among the enrolled 40 pairs of twins, LC3-II was significantly increased in the smaller twin placenta (FCR of 1.76 ± 1, p = 0.009) (Figure 1). The LC3-II FCR in MC pregnancies with sIUGR was significantly higher than that in MC pregnancies without sIUGR (FCR of 2.28 vs 1.04, p = 0.023). These results demonstrated that placental autophagic activity was significantly higher in the
abnormal UA Doppler, among the three groups of MC twin pregnancies (analysis of group III. The LC3-II FCR showed a significant difference in the three groups of MC twin pregnancies was related to the abnormal UA Doppler autophagic activity among the three groups of MC twins. This LC3-II FCR was significantly increased in the sIUGR twin with sIUGR group (mean ± standard deviation, 1.04 ± 0.28 versus 2.26 ± 0.31, p = 0.02)

**Table 1** Characteristics of MC twin pregnancies with and without sIUGR

<table>
<thead>
<tr>
<th></th>
<th>MC twin with sIUGR (n=21)</th>
<th>MC twin without sIUGR (n=19)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>33.2 ± 3.4</td>
<td>35.5 ± 2.5</td>
<td>0.037</td>
</tr>
<tr>
<td>Maternal age at delivery (years)</td>
<td>30.6 ± 4.3</td>
<td>29.4 ± 4.7</td>
<td>0.605</td>
</tr>
<tr>
<td>Birth weight of larger twin (g)</td>
<td>1969 ± 590</td>
<td>2345 ± 458</td>
<td>0.032</td>
</tr>
<tr>
<td>Birth weight of smaller twin (g)</td>
<td>1322 ± 497</td>
<td>2122 ± 258</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Birth weight discordance (%)</td>
<td>33.4 ± 12</td>
<td>8.8 ± 6.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Velamentous cord insertion in the sIUGR (smaller) twin, N (%)</td>
<td>8 (38.1)</td>
<td>2 (10.5)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data were presented as mean ± standard deviation.

MC, monochorionic; sIUGR, selective intrauterine growth restriction.

**Figure 1** Western blot of light chain 3 (LC3) protein of placenta tissue in twin pairs. Because of the prospective collection of twin placentas, we labeled delivery time by “A” and “B”. (A) First-delivery baby in monochorionic twins and (B) second-delivery baby in monochorionic twins. In twin I, A is the larger baby, and B is the smaller baby. In twins II to IV, A is the smaller baby, and B is the larger baby

sIUGR twin’s territory when using the MC cotwin without sIUGR as internal control (Figure 2).

The MC with sIUGR group was divided into two subgroups on the basis of the UA Doppler flow. Group I was sIUGR with abnormal UA Doppler flow, and group II was sIUGR without abnormal UA Doppler flow. AGA MC twins were defined as group III. The LC3-II FCR showed a significant difference among the three groups of MC twin pregnancies (analysis of variance, p < 0.05) (Figure 3). Post hoc comparison showed that LC3-II FCR was significantly higher in group I than in group III (Figure 3). This finding indicated that the difference in autophagic activity among the three groups of MC twin pregnancies was related to the abnormal UA flow.

**DISCUSSION**

In this study, increased placental autophagic activity was found in sIUGR MC twins compared with their AGA cotwins (used as controls). After taking into account UA Doppler data, we found that the strongest autophagic activity difference between the two twins’ territories was in the sIUGR twins with abnormal UA Doppler. To our knowledge, this is the first study to evaluate the autophagic activity in twin placentas. We provide strong evidence of increased placental autophagic activity in fetal growth restriction. The study also showed that abnormal UA flow is associated with increased placental autophagic activity.

Different autophagic activities of placentas have been reported from vaginal delivery and Cesarean section.12 All our placenta specimens came from Cesarean sections. MC twin fetuses grow in the same uterine environment, with identical genomic DNA, a perfect condition that excludes most confounding factors, such as maternal age, maternal nutrition, and gestational age. Although this is not the first study to evaluate autophagic activity in IUGR plenta, our results in an MC twin model are more convincing than singleton models because of the ideal environment that obviates the need to adjust for other factors.
Autophagy is stimulated by starvation and oxidative stress. Moreover, autophagy is thought to be associated with lipid and carbohydrate metabolism. In MC twins with sIUGR, the sIUGR fetus obtains less nutrition from the placenta. We have shown that MC sIUGR twins with abnormal UA Doppler have the least placenta share and lightest placenta weight. In this study, we found that LC3-II was the highest in MC with sIUGR group, which meant the placenta from the growth-restricted portion was highly autophagic. As we had hypothesized, the placenta with the least perfusion has the strongest autophagic activity. It is also compatible with prior research findings that indicated that the most common inducer of LC3-II is nutrition deprivation.

The incidence of abnormal cord insertion, such as VCI, is higher in twin pregnancies than in their singleton counterpart. VCI is thought to be associated with preterm labor, low birth weight, or small for gestation age. In this study, VCI was more frequently seen in the MC twin with sIUGR. VCI may be related to a smaller placenta and relatively poor perfusion. Consequently, the increased autophagic activity in MC with sIUGR group may also be correlated with abnormal cord insertion. Whether there is a causal link would require further research to address this issue.

The LC3 maturation (LC3-I and II) has been reported as an autophagic marker. Previous studies have shown that nutrition deprivation stimulates autophagy. Further studies are needed to investigate whether intrauterine autophagic activity has an effect on subsequent child development.

**WHAT DOES THIS STUDY ADD?**

- Autophagic activity is increased in the placental territory of selective intrauterine growth-restricted monochorionic twins, and the phenomenon is aggravated in the presence of abnormal umbilical artery blood flow at Doppler.

**REFERENCES**