Why are levels of maternal microchimerism higher in type 1 diabetes pancreas?

Berendine vanZyl,¹ Raquel Planas,² Yi Ye,¹ Alan Foulis,³ Ronald R. de Krijger,⁴ Marta Vives-Pi² and Kathleen M. Gillespie^{1,*}

¹Diabetes and Metabolism; Department of Clinical Science at North Bristol; University of Bristol; Bristol, UK; ²LIRAD-BST; Fundació Institut d'Investigació en Ciències de la Salut Germans; Badalona, Spain; ³Department of Pathology; Royal Infirmary; Glasgow, UK; ⁴Department of Pathology; Josephine Neffkens Institute; Erasmus MC-University Medical Center Rotterdam; Rotterdam, The Netherlands

Key words: maternal microchimerism, type I diabetes, islet beta cells, endocrine, exocrine

Maternal microchimerism (MMc) results from transfer of maternal cells to the fetus in pregnancy. These cells have been shown to persist into adulthood in healthy individuals and an increased frequency of MMc has been associated with autoimmune disease. Female (presumed maternal) islet beta cells have recently been identified at higher levels in pancreas from a child with T1D compared to three controls. There was, however, no evidence that these cells were the targets of autoimmune attack. The aim of this study was to analyze well-characterized T1D pancreases encompassing a spectrum in age at diagnosis, and duration of diabetes, for the presence of maternal microchimerism compared to control pancreases.

Pancreas samples were available from six males with T1D and four male controls. Fluorescent-labeled probes were used to detect X and Y chromosomes. At least 1,000 cells, usually 4,000–8,000 cells underwent confocal imaging for each pancreas. The frequency of MMc was higher in T1D pancreases (range 0.31–0.80%, mean 0.58%) than in controls (0.24–0.50%, mean 0.38%) ($p = 0.05$). Intriguingly, clusters of 2–3 MMc were occasionally found in the pancreases, particularly T1D pancreases, suggesting replication of these cells. Concomitant FISH and immunofluorescence staining for insulin or CD45 was performed to phenotype cells of maternal origin. Insulin positive and insulin negative MMc were identified indicating that MMc contribute to the exocrine and endocrine compartments. No CD45 positive MMc were observed. These data confirm the presence of maternal cells in human pancreas and support previous observations that levels of MMc are higher in T1D pancreas compared to controls. MMc do not appear to be immune effector cells and those that stain positive for insulin within intact islets in T1D tissue appear healthy with no evidence that they are the focus of immune attack. This study adds support to the hypothesis that maternal stem cells have the capacity to cross the placental barrier and differentiate into both endocrine and exocrine cells but more detailed characterization of MMc in the pancreas is required.

Introduction

Maternal microchimerism (MMc, the harboring of a small number of maternal cells, or DNA by her offspring) was recognized in children with severe combined immunodeficiency more than 30 years ago¹ and has been found to persist into adult life in healthy subjects.² Most studies of maternal microchimerism have focused on analysis of hematopoietic cells but it is increasingly clear from human³ and animal studies⁴ that microchimeric cells also exist in other tissues including the liver, heart and brain.

The persistence of maternal cells implies that the offspring is tolerant to a low level of genetically distinct antigen but maternal microchimerism has also been associated with autoimmune diseases. For example, maternal microchimerism was found to be significantly more frequent in patients with systemic

sclerosis (SSc) (72%) versus controls (22%).⁵ Similarly, when peripheral blood and muscle biopsies from children with juvenile dermatomyositis (JDM) were examined, the frequency of maternal microchimerism was significantly increased in the blood and muscle biopsied from children with JDM than from unrelated controls and unaffected siblings.⁶

We previously identified female cells (presumed maternal) in autopsy pancreas from young males, with and without type 1 diabetes, that appear to produce insulin.⁷ These cells could potentially be the target of the immune response in type 1 diabetes or play a role in attempted regeneration of damaged tissue. The aim of this study was to establish the levels of maternal microchimerism in six pancreas samples from individuals with well characterized type 1 diabetes at different stages of the disease process compared with four healthy controls, and where possible to phenotype these MMc using insulin as a marker of beta cells

*Correspondence to: Kathleen M. Gillespie; Email: K.M.Gillespie@bristol.ac.uk

Submitted: 06/22/10; Revised: 08/27/10; Accepted: 10/08/10

Previously published online: www.landesbioscience.com/journals/chimerism/article/13981

DOI: 10.4161/chim.1.2.13891

Figure 1. Consecutive confocal images of a maternal microchimeric cell (MMc-indicated by the arrow) from a control fresh frozen tissue section (31 years). The X chromosome is shown by a red dot, the Y chromosome by a green dot and the nucleus with DAPI (blue).

and CD45 as a marker of lymphocytes to help clarify their role in health and disease.

Results

MMc identification. To ensure that each MMc observed was unequivocally an XX cell in XY tissue and not the result of non-disjunction (XXY) where the Y chromosome signal was hidden, the observed XX cells underwent confocal imaging through the nucleus as demonstrated in **Figure 1**. Video images are available for all tissue analyzed. In this project, cells with XXY or YYX signals were observed but were very rare, except for an 89 year-old recent onset T1D patient where 0.40% of cells were XXY and 0.56% were YYX. Polyploidy was therefore observed more often in this older T1D patient.

Analysis of MMc frequency in pancreatic tissue. MMc frequency was analyzed in tissue that had undergone FISH without concomitant immunofluorescence. MMc were identified in all pancreatic sections that underwent confocal examination. More than 1,000 cells were counted in each sample and where tissue was available more than 4,000 cells were counted. The results obtained are shown in **Table 1** and represented in **Figure 2**. Differences between MMc levels in the T1D and control tissue analyzed using a Student's t test reached statistical significance ($p = 0.05$). Unexpectedly as shown in **Figure 3A and B**, MMc were sometimes observed in small groups or clusters particularly in T1D tissue.

MMc phenotyping in human pancreas. *Insulin.* X and Y chromosome FISH with concomitant insulin immunofluorescence was carried out in all control and T1D samples. Good quality staining of islets was observed in all controls and in recent onset

T1D samples 2–4 while sporadic insulin staining was observed in the tissue from longstanding T1D samples. A typical MMc within an insulin stained (FITC) islet from control sample 3 (an individual who died at age 21 years) is shown in **Figure 4A** and from T1D sample 2 (who died at 89 years) in **Figure 4B**. The frequency of maternal microchimeric cells in the islets was compared to the frequency of maternal microchimeric cells in the pancreas overall in control sample 3 and T1D sample 2. It was found that the frequency of insulin staining maternal microchimeric cells was increased in islets; 1.33% and 1.49% compared to 0.50% and 0.54% respectively, in the pancreatic tissue overall.

CD45. X and Y chromosome FISH with concomitant insulin immunofluorescence CD45 staining was carried out in T1D sample 5 and control sample 1. CD45+ve cells were identified only rarely within the healthy control pancreatic tissue (3 of 542 cells examined) and at a higher level in the T1D tissue (9 of 437 individual cells counted) with occasional clusters of CD45 positive cells consistent with autoimmune lymphocytic infiltration of islets. No convincing CD45 positive MMc were observed in either control or T1D tissue. A typical image is shown in **Figure 5** where 3 CD45+ve FITC stained cells lie close to an MMc. Although the X and Y signals are covered by the CD45 signal, by attenuating the FITC signal, these cells appear to contain both X and Y chromosomes.

Discussion

This study of 6 T1D and 4 control pancreas samples confirms the presence of maternal cells in human pancreas. Detailed analysis of MMc frequency in these samples using confocal imaging of at least 1,000 cells per section resulted in identification of MMc in all sections. Analysis of MMc levels indicated that significantly more MMc were identified in T1D as compared to control tissue and this did not appear to correlate with age or time from diagnosis. In order to identify endocrine versus exocrine pancreatic tissue, immunofluorescence staining for insulin was employed. In all pancreas samples analyzed MMc were identified within islets that appear to be insulin positive but non-insulin staining MMc were also observed in the exocrine tissue. Detailed counting of MMc in islets in one control and one T1D tissue suggest that levels of MMc are higher within islets.

Immunofluorescent staining for the lymphocytic marker CD45 on one T1D and one control sample did not result in visualization of any CD45 positive MMc in pancreatic tissue.

Table 1. Levels of maternal microchimerism in T1D and control pancreas

Sample	Age at death (years)	Duration of diabetes	Total cell count	FISH success rate (%)	Number of MMc by confocal imaging	MMc frequency
T1D 1	65 years	Longstanding	3186	74	10	0.31
T1D 2	89 years	Recent	6472	55	35	0.54
T1D 3	11 years	Recent	7194	64	39	0.56
T1D 4	16 years	9 months (Recent)	1094	63	7	0.64
T1D 5	29 years	8 years (Longstanding)	4608	62	37	0.8
T1D 6	26 years	10 years (Longstanding)	5281	73	33	0.62
Control 1	10 days		6698	81	24	0.24
Control 2	1 year		2487	74	12	0.48
Control 3	21 years		4176	49	21	0.5
Control 4	31 years		8493	76	25	0.29

MMc frequency was calculated by counting the number of XX cells in male tissue using confocal microscopy.

Figure 2. MMc frequency (%) in six T1D (closed circles) and four controls (closed squares). The Mean and SD are indicated for each group ($p = 0.05$).

This suggests that MMc in pancreas are not effector cells of the immune system in T1D although analysis of a larger number of samples will be required to confirm this.

This study has lead to some intriguing, albeit anecdotal, observations of MMc in pancreas. For instance although MMc are rare, generally occurring at a frequency of less than 40 MMc per 1,000 cells, they appear more often than might be expected close to or within islets and often occur in small groups or clusters of two or three cells which may suggest replication.

One weakness of this study is that although staining for insulin was possible in combination with FISH analysis, other specific nuclear markers of beta cells (pdx-1, mafA and neuro D) did not work well with concomitant FISH. It is difficult therefore to say definitively that the insulin positive MMc observed in this study are beta cells. Further studies are therefore required to phenotype MMc in islets in more detail.

These data do raise the questions: What are these cells? Why are they present in the pancreas and why do they appear to be present at higher levels in T1D pancreas? Maternal cells could be effectors of the autoimmune response as has been proposed in children with dermatomyositis⁸ but our data suggest that this is not the case in T1D. MMc in pancreas however could be targets

Figure 3. (A) Two adjacent MMc (indicated by arrows) in pancreatic T1D tissue sample 1 (65 year old with longstanding diabetes); (B) four maternal cells (indicated by arrows) in one field from an 11 year old recent-onset T1D patient (T1D sample 3). The X chromosome is shown by a red dot and the Y chromosome by a green dot. The cytoplasmic green staining represents insulin and the nucleus is stained with DAPI (blue).

of the autoimmune response. It might be hypothesized that levels of MMc in pancreas above a certain threshold could contribute to triggering of the autoimmune process. As yet however there are few data to support this model. Some have suggested that fetal microchimeric cells may play a beneficial role contributing to regeneration of damaged tissue.⁹ In the normal pancreas, beta cell replication occurs in infancy but decreases thereafter.¹⁰ There has been a recent suggestion that islet cell proliferation is increased in patients with recent onset diabetes.¹¹ Indeed a marked increase in the frequency of beta cell replication has already been reported in tissue from the 89 year-old T1D patient which has also been included in our study.¹²

Targeted regeneration of beta cells offers a potential strategy to prevent T1D. Regeneration of beta cells is therefore an area of major active investigation, with recent studies reporting differentiation of pancreatic and nonpancreatic progenitors and replication of existing islet beta cells. Lineage tracing studies suggest that replication of pre-existing beta cells is the primary mechanism of postnatal growth and regeneration of in mice.¹³ Other sources of beta cells have been proposed, including progenitor

Figure 4. (A) a low and high power image of an MMc indicated by the arrow within an insulin stained islet from a healthy control section; (B) two islets from T1D sample 2 (recent onset 89-year-old) with an MMc in each islet; (C) an insulin-ve MMc outside an insulin stained islet. The X chromosome is shown by a red dot and the Y chromosome by a green dot. The cytoplasmic green staining represents insulin and the nucleus is stained with DAPI (blue).

cells residing in the exocrine ducts,¹⁴ bone marrow stem cells¹⁵ and dedifferentiation of beta cells into replicating epithelial cells before redifferentiation.¹⁶ There is increasing evidence from animal models that the damaged pancreas has the capacity to regenerate by injury induced activation of stem cells.¹⁷ The model that maternal progenitors home to the site of injury and contribute to islet cell proliferation in T1D is attractive but requires mechanistic proof.

Methods

Pancreatic autopsy tissue sample. Ten male pancreas samples were examined: six from individuals with T1D and four healthy controls. T1D samples 1 and 2 were obtained from Dr. Peter Butler, UCLA, USA with IRB permission from UCLA. Both cases, one from a 65 year-old with longstanding type 1 diabetes and the other from an 89 year-old with recent onset diabetes were obtained as a result of pancreatic surgery where a normal piece of tissue was retained for research. Sample 2 has been described in

detail previously.¹² T1D sample 3 obtained from Dr. Alan Foulis, Department of Pathology, Glasgow Royal Infirmary, was from an 11-year-old boy with recent onset type 1 diabetes who died as a complication of ketoacidosis.¹⁸ T1D samples 4–6 were obtained from Dr. Martha Vives-Pi Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain.¹⁹ Sample 4 was from an 11 year-old with T1D for nine months and sample 5 from a 29 year-old with T1D of eight years duration. Both died as a result of brain edema. The sixth sample was from a 26 year-old with T1D of 10 years duration who died as a result of head trauma. T1D samples 1–3 were paraffin embedded tissue while samples 4–6 were fresh frozen.

Controls 1 and 2 (who died at ten days and one year, respectively) were obtained from Dr. Ronald de Kriger (Department of Pathology, Erasmus MC-University Medical Centre, Rotterdam, Netherlands)²⁰ while control samples 3 and 4 who died at 21 and 31 years were also obtained from Dr. Martha Vives-Pi (Fundació Institut d'Investigació en Ciències de la Salut

Germans Trias i Pujol, Badalona, Spain). Control samples 1 and 2 were paraffin embedded while control samples 3 and 4 were fresh frozen tissue.

Full consent was available for all samples and appropriate local ethical approval was obtained for all tissues (National Research Ethics Service 04/Q2002/35).

Strategy. Our approach was first to stain the X and Y chromosome using fluorescent in situ hybridization (FISH) and then to carry out concomitant immunofluorescence to identify the antigen(s) of interest. Maternal microchimeric cells were identified, quantified, and characterized using confocal microscopy.

FISH with concomitant immunofluorescence. Paraffin embedded blocks were cut into 4 μ m sections on positively charged slides prior to FISH/immunofluorescence analysis. Briefly, sections were initially deparaffinized in xylene, dehydrated in 100% ethanol, and washed in 0.2 N HCl. The sections were then washed in water before immersing them in heat pre-treatment solution with disodium EDTA (Zymed SPOT-Light® Tissue Heat Pretreatment Solution, Zymed® Laboratories, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA, 92008) at 95°C for 100 minutes. After washing with water, proteins were digested for 60 minutes using Pepsin A (Digest-All 3 protease) (Zymed SPOT-Light® Tissue Pretreatment kit, Zymed® Laboratories, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA, 92008). The sections were then fixed in 1% formaldehyde solution, dehydrated through an ethanol series and air dried. The FISH probes (Vysis CEP® X Spectrum Orange™/Y SpectrumGreen™ Direct Labeled Fluorescent DNA Probe, Abbott Molecular Inc., Des Plaines, IL 60018) were prepared according to the manufacturer's recommendation, and then applied on the tissue sections. DNA was denatured at 73°C for 10 minutes and then renatured with FISH probes by incubating overnight at 42°C. The following day, after post-hybridization washes, immunofluorescence was started. Non-specific binding was blocked by incubating the slides with normal blocking serum for 60 minutes. Subsequently slides were incubated with primary antibody (Insulin—Guinea Pig Polyclonal, CD45—Mouse monoclonal, all from Dako UK Ltd., Cambridge House, St. Thomas Place, Ely, Cambridgeshire CB7 4EX) for 4 hours at 37°C, washed with 1 x PBS/0.05% Tween-20, and then incubated with fluorescent secondary antibodies for 2 hours at 37°C. For negative control slides, primary antibody was replaced by normal blocking serum. Sequential staining was performed for labeling double antigens. The sections were then dehydrated through an ethanol series, air dried, and mounted with 4',6-diamidino-2-phenylindole (DAPI)

Figure 5. X and Y chromosome FISH with concomitant immunofluorescence for CD45 (FITC) in a control pancreas section. 3 CD45 cells are indicated with a CD45-ve MMc close by.

counter stain and mounting medium (VECTOR laboratories Inc., 30 Ingold Road, Burlingame, CA, 94010, USA).

7 μ m fresh frozen tissue sections (stored at -80°C) were air dried prior to FISH probe hybridization, immunofluorescence and analysis was carried out as above.

Analysis. All sections were analyzed under a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope. Images were taken by using a 63x magnification (oil) lens and 2x digital zoom. Cells were analyzed by assigning them to one of four categories: X, Y, XY or XX. FISH success rate (frequency of nuclei with both the X and Y chromosome observable) was determined in each sample. An unpaired student's t-test was used to determine statistical differences in MMc frequency between T1D and control groups.

Acknowledgements

The authors are grateful to the Juvenile Diabetes Research Foundation, the European Foundation for the Study of Diabetes and Diabetes UK for funding.

References

- Pollack MS, Kapoor N, Sorell M, Kim SJ, Christiansen FT, Silver DM, et al. DR-positive maternal engrafted T cells in a severe combined immunodeficiency patient without graft-versus-host disease. *Transplantation* 1980; 30:331-4.
- Maloney S, Smith A, Furst DE, Myerson D, Rupert K, Evans PC, Nelson JL. Microchimerism of maternal origin persists into adult life. *J Clin Invest* 1999; 104:41-7.
- Stevens AM, Hermes HM, Kiefer MM, Rutledge JC, Nelson JL. Chimeric maternal cells with tissue-specific antigen expression and morphology are common in infant tissues. *Pediatr Dev Pathol* 2009; 12:337-46.
- Chen CP, Lee MY, Huang JP, Aplin JD, Wu YH, Hu CS, et al. Trafficking of multipotent mesenchymal stromal cells from maternal circulation through the placenta involves vascular endothelial growth factor receptor-1 and integrins. *Stem Cells* 2008; 26:550-61.
- Lambert NC, Erickson TD, Yan Z, Pang JM, Guthrie KA, Furst DE, Nelson JL. Quantitation of maternal microchimerism by HLA-specific real-time polymerase chain reaction: studies of healthy women and women with scleroderma. *Arthritis Rheum* 2004; 50:906-14.
- Reed AM, Picornell YJ, Harwood A, Kredich DW. Chimerism in children with juvenile dermatomyositis. *Lancet* 2000; 356:2156-7.
- Nelson JL, Gillespie KM, Lambert NC, Stevens AM, Loubiere LS, Rutledge JC, et al. Maternal microchimerism in peripheral blood in type 1 diabetes and pancreatic islet beta cell microchimerism. *Proc Natl Acad Sci USA* 2007; 104:1637-42.
- Reed AM, McNallan K, Wettstein P, Vehe R, Ober C. Does HLA-dependent chimerism underlie the pathogenesis of juvenile dermatomyositis? *J Immunol* 2004; 172:5041-6.
- O'Donoghue K, Sultan HA, Al-Allaf FA, Anderson JR, Wyatt-Ashmead J, Fisk NM. Microchimeric fetal cells cluster at sites of tissue injury in lung decades after pregnancy. *Reprod Biomed Online* 2008; 16:382-90.

10. Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, et al. Beta cell replication is the primary mechanism subserving the postnatal expansion of beta cell mass in humans. *Diabetes* 2008; 57:1584-94.
11. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Evidence of increased islet cell proliferation in patients with recent-onset type 1 diabetes. *Diabetologia* 2010; 53:2020-8.
12. Meier JJ, Lin JC, Butler AE, Galasso R, Martinez DS, Butler PC. Direct evidence of attempted beta cell regeneration in an 89-year-old patient with recent-onset type 1 diabetes. *Diabetologia* 2006; 49:1838-44.
13. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic β -cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 2004; 429:41-6.
14. Bonner-Weir S, Toschi E, Inada A, Reitz P, Fonseca SY, Aye T, Sharma A. The pancreatic ductal epithelium serves as a potential pool of progenitor cells. *Pediatr Diabetes* 2004; 5:16-22.
15. Kodama S, Kühtreiber W, Fujimura S, Dale EA, Faustman DL. Islet regeneration during the reversal of autoimmune diabetes in NOD mice. *Science* 2003; 302:1223-7.
16. Weinberg N, Ouziel-Yahalom L, Knoller S, Efrat S, Dor Y. Lineage tracing evidence for in vitro dedifferentiation but rare proliferation of mouse pancreatic beta-cells. *Diabetes* 2007; 56:1299-304.
17. Xu X, D'Hoker J, Stangé G, Bonn S, De Leu N, Xiao X, et al. Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell* 2008; 25:197-207.
18. Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25 year review of deaths in patients under 20 years of age in the United Kingdom. *Diabetologia* 1986; 29:267-74.
19. Planas R, Carrillo J, Sanchez A, de Villa MC, Nuez F, Verdaguer J, et al. Gene expression profiles for the human pancreas and purified islets in type 1 diabetes: new findings at clinical onset and in long-standing diabetes. *Clin Exp Immunol* 2010; 159:23-44.
20. Korpershoek E, Leenen PJ, Drexhage HA, De Krijger RR. Cellular composition of pancreas-associated lymphoid tissue during human fetal pancreatic development. *Histopathology* 2004; 45:291-7.