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Embryonic stem cells markers are present within rabbit atherosclerotic plaques

Anthony Zulli[1,3], Brian F. Buxton[2], M. Jane Black[4] and David L. Hare[1,3]

[1]Departments of Cardiology, [2]Cardiac Surgery and [3]Medicine, University of Melbourne, Austin Health, Australia and [4]Department of Anatomy and Cell Biology, Monash University, Clayton, Australia

Summary. Recent evidence suggests that smooth muscle cells within atherosclerotic plaques originate from vascular progenitor cells. We have previously shown that smooth muscle cells and macrophages present within rabbit atherosclerotic plaques are positive for factors of the renin angiotensin and nitric oxide systems as well as the hematopoietic stem-cell marker CD34 and the pan-leukocyte marker CD45. To explore the idea that these cells are of primitive types, immunohistochemistry was used to identify pluripotent embryonic stem cells (ESC) markers (Oct-4, SSEA1,3,4, TRA1-60, 81) in these plaques and to compare these to intimal thickening. Objective: To immunolocalise ESC markers in rabbit aortic intimal thickening and atherosclerotic plaques. Design: New Zealand White rabbits were fed either a control (Con) diet, 0.5% cholesterol (Chol) or 1% methionine (Meth) for 12 weeks. Animals were perfusion fixed, aortae excised and processed for paraffin. Immunohistochemistry was performed by standard techniques. Results: Oct-4, SSEA 1, 3 and 4, TRA-1-60 and TRA-1-81 were all present within in atherosclerotic plaques. However, some cells were not positive for TRA-1-60 and TRA-1-81. In fact, positive TRA-1-81 macrophages were uncommon, and positive TRA-1-81 smooth muscle cells were rare. Intimal thickening in Meth did not show any TRA-1-81 positive cells Conclusions: Macrophages and smooth muscle cells within atherosclerotic plaques express markers of ESC. These results suggest that cells within these plaques are primitive and might differentiate into other types of cells.

Offprint requests to: Dr. Anthony Zulli, Vascular Laboratory, Department of Cardiology, Austin Health, Heidelberg, Australia. e-mail: azulli@unimelb.edu.au

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Introduction

Pluripotent embryonic stem cells (hES) differentiate into a wide range of cell types. These cells are identified by the expression of the stem cell transcription factor, Octomer-4 (Oct-4) (Thomson et al., 1998; Reubinoff et al., 2000), the stage-specific embryonic antigens (SSEA) 3 and 4 (Thomson et al., 1998; Reubinoff et al., 2000), as well as the keratan sulphate-associated antigens (tumor rejection antigen), TRA-1-60 and TRA-1-81, but not SSEA 1(Andrews et al., 1984; Badcock et al., 1999). As ES cells differentiate, they down regulate SSEA3, SSEA4, TRA-1-60 and TRA-1-81, but up regulate SSEA 1 (Draper et al., 2002).

Recent evidence suggests that ES cells can differentiate into vascular cells (Hirashima et al., 2003), and we have previously shown that smooth muscle cells and macrophages within atherosclerotic plaques express the hematopoietic stem-cell marker CD 34 (Zulli et al., 2005), as well as factors of the renin-angiotensin and nitric oxide systems (Zulli et al., 2006a,b). For example, we co-localised in smooth muscle cells and macrophages angiotensin II type 2 receptors and angiotensin converting enzyme 2 (Zulli et al., 2006a), endothelial nitric oxide synthase and caveolin-1 (Zulli et al., 2006b), and separately inducible nitric oxide synthase and superoxide dismutase (Zulli et al., 2003). Taken together, these results suggest that the endothelial progenitor cells within plaques (CD 34+) are primitive cells that express several factors of the renin-angiotensin and nitric oxide systems.

Therefore, as primitive embryonic stem cells give rise to the hematopoietic stem-cell line (CD 34+), we sought to investigate whether intimal thickening, caused by high dietary methionine, or atherosclerotic plaques, caused by high dietary cholesterol, expressed these early

ES markers.

Materials and methods

Male New Zealand White rabbits at three months of age were divided into three groups, a control group (con), that was fed a normal rabbit chow diet; an atherosclerotic group (Chol), that received a normal rabbit chow diet supplemented with 0.5% cholesterol, and an intimal hyperplastic group (Meth), that received a normal rabbit chow diet supplemented with 1% methionine. The animals were housed in individual cages and maintained at a constant temperature of approximately 21°C. Food and water were supplied *ad libidum*. The animals were fed their respective diet for twelve weeks. The experiments were carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (6th Edition, 1997). The animals were perfusion-fixed as previously described in our laboratory (Zulli et al., 2003, 2005, 2006a,b).

Immunohistochemistry

Oct-4, SSEA1, SSEA3, SSEA4, TRA-1-60, TRA-1-81, monoclonal antibodies were purchased from Chemicon International, negative control antibody was purchased from DAKO Corporation, California. All primary antibodies were diluted 1:100 and incubated

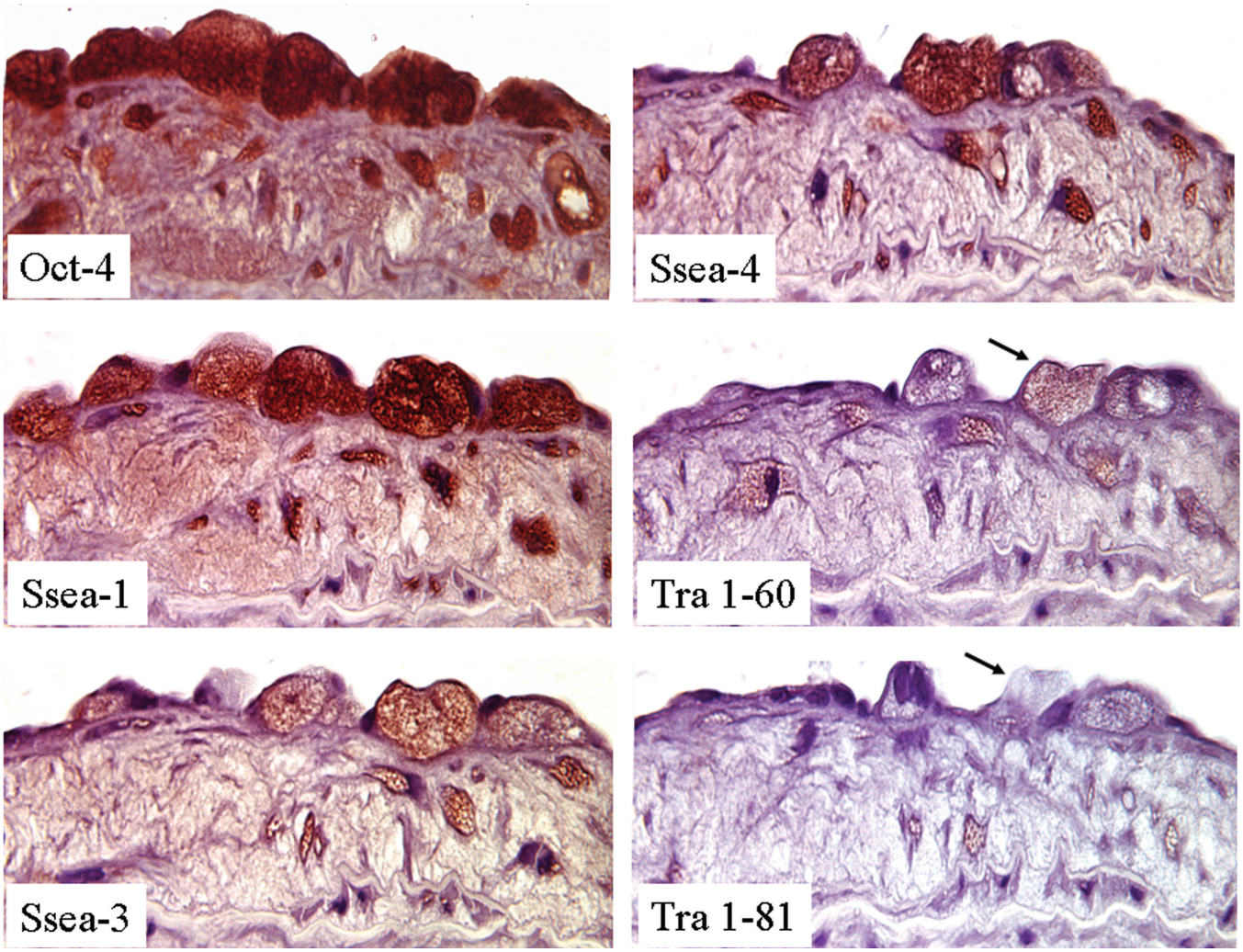

Fig. 1. Photomicrograph of serial adjacent sections of the thoracic aorta of rabbits fed high dietary cholesterol. A magnification of atherosclerotic plaques is shown. Both macrophages and smooth muscle cells within plaques show positive immunoreactivity for Oct-4, SSEA-1,3 and 4. However, not all cells within plaques were positive for Tra-1-81 and Tra-1-60 (arrow). Some macrophages and smooth muscle cells were either positive for Tra-1-81 or Tra-1-60 (arrow). x 1,000

overnight. For the negative, a monoclonal antibody to *Aspergillus niger* glucose oxidase was diluted 1:20 and incubated overnight. Then, immunohistochemistry was performed as previously described in our laboratory (Zulli et al., 2003, 2005, 2006a,b) using a commercially available immunohistochemistry kit and immunoquantification as established in our laboratory (Zulli et al., 2006b).

Results

In the thoracic aorta of rabbits fed high dietary cholesterol, cells that stained positive for ESC markers were very common not only in plaques, but also on the endothelium overlying neo-intima and normal wall. Oct-4, SSEA-1,3 and 4 all showed similar binding characteristics (Figs. 1-3). However, not all cells within plaques were positive for Tra-1-81 and Tra-1-60 (Fig. 1). Some macrophages and smooth muscle cells were either positive for Tra-1-81 or Tra-1-60 (Fig. 1, arrow).

In the control vessels, endothelial cells that were positive for Oct-4, SSEA-1,3 and 4 were very rare in the endothelial layer overlying normal vessel and intimal thickening (Fig. 4a), and no positive immunostaining for Tra-1-81 (Fig. 4b) or Tra-1-60 were observed.

In the thoracic aorta of rabbits fed high dietary methionine, vessels, Oct-4 positive cells increased 5 fold ($p<0.05$) in the endothelial layer overlying neo-intima formation, but were rare in disease free areas. SSEA-1,3 and 4 immunopositive cells were similar to Oct-4. Interestingly, there were no Tra-1-81 or Tra-1-60 positive cells in the endothelia overlying intimal thickening or disease free areas

In the thoracic aorta of rabbits fed high dietary cholesterol, Oct-4 binding in the endothelium overlying normal media increased 10 fold ($p<0.05$) and 23 fold ($p<0.05$) in endothelium overlying intimal thickening. Similar binding characteristics were observed with SSEA-1,3 and 4. In stark contrast to the blood vessels in the control or Meth group, Tra-1-81 and Tra-1-60 positive cells were abundant throughout the blood vessels in this group. Indeed, Tra-1-81 positive immunoreactivity increased 10 fold ($p<0.05$) in endothelium overlying normal media and 9 fold ($p<0.05$) in endothelium overlying intimal thickening (Fig. 4b).

In all blood vessels studied, no positive ESC cells were observed in the medial layer. In the adventitia, positive ESC cells were commonly found in all groups. For example, OCT-4, SSEA-1, SSEA3 and SSEA4 positive cells were observed, however TRA-1-60 and

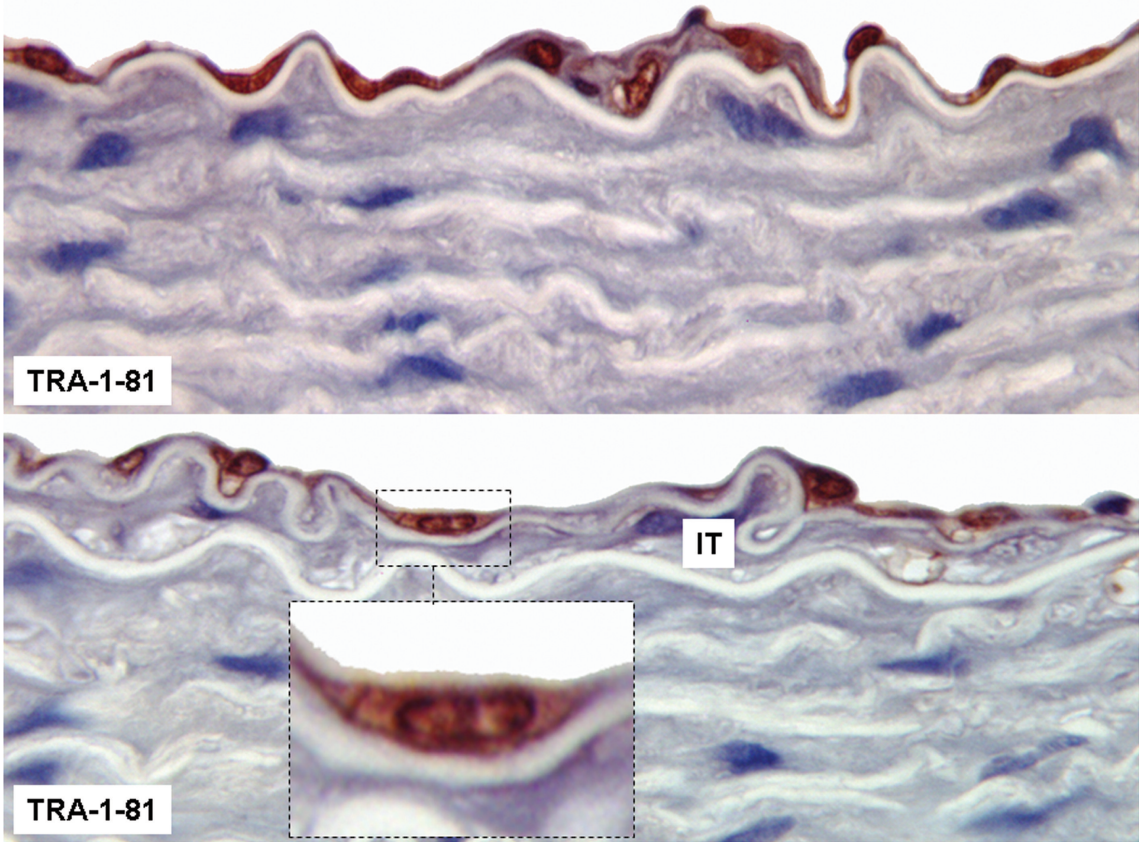

Fig. 2. Photomicrograph of the thoracic aorta of rabbits fed high dietary cholesterol. A magnification of normal endothelium and endothelium overlying neo-intimal thickening is shown. Single cells, possibly smooth muscle cells show positive immunoreactivity for Tra-1-81. These cells were not observed in control or rabbits fed 1% methionine. x 2,000

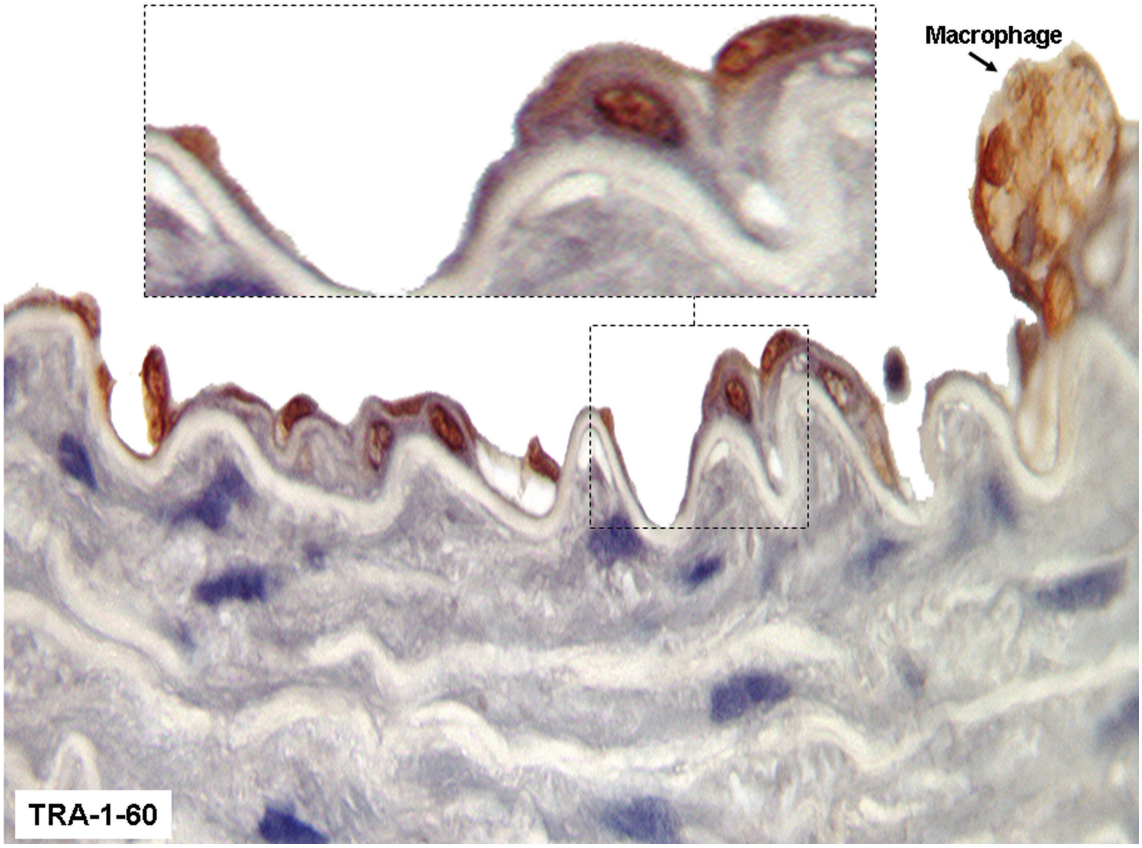

Fig. 3. Photomicrograph of the thoracic aorta of rabbits fed high dietary cholesterol. A magnification of normal endothelium and endothelium overlying neo-intimal thickening is shown. Single cells, possibly smooth muscle cells, as well as a binding macrophage show positive immunoreactivity for Tra-1-60. These cells were not observed in control or rabbits fed 1% methionine. x 2,000

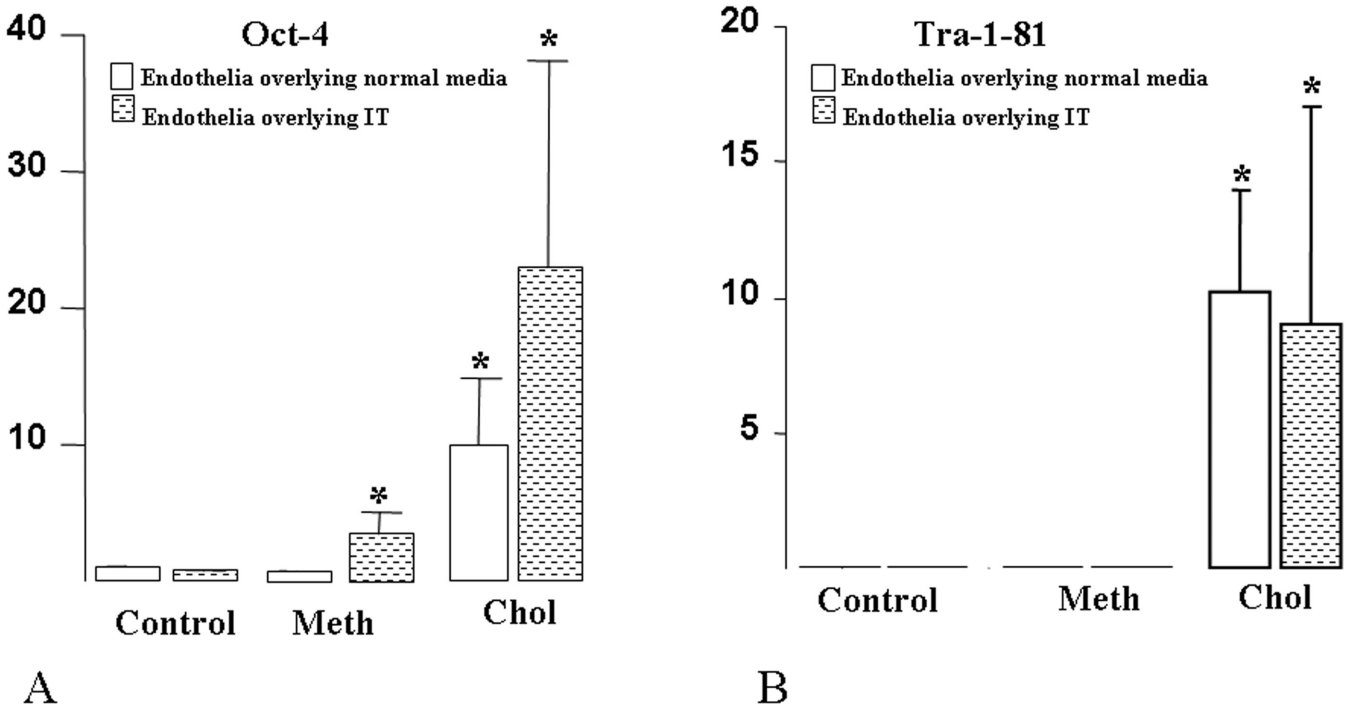

Fig. 4. Bar graph showing the immunoquantification of Oct-4 **(A)** and Tra-1-81 **(B)** in the endothelium of rabbit thoracic aorta. Oct-4 immunoreactivity was increased in experimental animals, but not in control. In the thoracic aorta of rabbits fed high dietary methionine, vessels, Oct-4 positive cells increased 5 fold ($p<0.05$) in the endothelial layer overlying neo-intima formation and increased 10 fold ($p<0.05$) and 23 fold ($p<0.05$) in endothelium overlying normal wall and intimal thickening in the cholesterol fed group. Tra-1-81 positive cells were only increased ($p<0.05$) in the endothelium of the cholesterol fed group.

TRA-1-81 positive cells were rarely seen.

Discussion

The major findings in this investigation are that macrophages and smooth muscle cells in atherosclerotic plaques are positive for the primitive embryonic stem cell markers. Such cells were present both overlying and within atherosclerotic plaques. Furthermore, Tra-1-81 and 1-60 are only present in cholesterol fed animals, indicating a possible role for these specific cells in atherogenesis.

We have previously reported that these cells also react with antibodies to alpha SMC actin, RAM-11 (macrophage), prolyl-4- hydroxylase and the pan-leukocytic marker, CD45 and the hematopoetic cell marker, CD 34 (Zulli et al., 2005). Here we provide further evidence that these cells within plaques are of primitive origin, and as such suggest that these cells could further differentiate into other cell types. This view is supported by Vrana and collegues, whom have recently created monkey pluripotent line of stem cells that are positive for Oct-4, SSEA-4,TRA 1-60 and TRA 1-81, those cells can differentiate in vitro into dopaminergic and serotonergic neurons, contractile cardiomyocyte-like cells, smooth muscle, ciliated epithelia, and adipocytes (Vrana et al., 2003).

Oct-4 is expressed in totipotent embryonic cells. When embryonic stem cells are triggered to differentiate, Oct-4 is downregulated. Recent experiments indicate that an Oct-4 expression level of roughly 50%-150% of the endogenous amount in embryonic stem cells is permissive for self-renewal and maintenance of totipotency. Thus a critical amount of Oct-4 is required to sustain stem-cell self-renewal, and up- or downregulation induces divergent developmental programmes (Pesce and Scholer, 2001). Here, we have shown that Oct-4 positive immuoreactivity is scarcely present in normal rabbit thoracic aorta, but significantly increased in endothelium overlying neo-intima formation during high dietary methionine or cholesterol. In fact, Oct-4 positive cells were also present in the endothelium overlying normal wall in the cholesterol fed group. These results clearly indicate a pluripotent role for these positive cells in the aorta.

In this study, we have observed TRA (tumor rejection antigen) positive cells only in the aorta of cholesterol fed groups. Tumor rejection antigen is an operational term describing how well an immune response elicited against a tumor antigen will impact on tumor growth. In this case, it is unknown what effect the TRAs have on atherogenesis, but it is clear that they are present in undifferentiated human embryonal carcinoma (EC) cells (Andrews, 1984; Andrews et al., 1996), indicating a possible link between carcinogenesis and atherogenesis.

Similarly, stage-specific embryonic antigens are also expressed by pluripotent cells. In particular, SSEA-1 can be specifically lost during the differentiation of mouse ES cells (Solter et al., 1979) or upregulated during human ES differentiation (Andrews, 1984; Fenderson et al., 1987). The regulation of SSEA-1 expression in rabbit ES cells is unknown, although here we show that SSEA-1 is present in atherosclerotic plaques in the same cells that also express all other markers. Whether SSEA-1 is increased or decreased in these tissues remains to be elucidated.

The conventional view for neo-intimal formation is now being revamped, as it is becoming evident that the majority of cells present in atherosclerotic tissues are from bone marrow origin and not from SMCs derived from the adjacent medial layer. For example, Sata and colleagues have shown that most of the alpha SMC actin positive smooth muscle cells in models of post-angioplasty restenosis, graft vasculopathy and hyperlipidemia-induced atherosclerosis, originated from bone marrow cells (Sata et al., 2002). We support this view, by showing that the cells that are present in neo-intimal thickening and atherosclerotic plaques are positive for white blood cell markers (Zulli et al., 2005) embryonic stem cell factors.

In conclusion, we report for the first time, that the previously identified macrophages and smooth muscle cells that are present within atherosclerotic plaques of rabbits fed high dietary cholesterol bind to embryonic stem cell markers and are thus primitive and pluripotent cells.

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